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Recent Advances in Petroleum Microbiology

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INTRODUCTION

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most notably complexing vanadium and nickel. Petroleum recovered from different reservoirs varies widely in compositional and physical properties. Long recognized as substrates supporting microbial growth (92, 580), these hydrocarbons are both a target and a product of microbial metabolism (169). Biodegradation by microorganisms modifies waxy crude oils in beneficial ways, but conditions for down-hole applications require the use of thermophiles, resistant to organic solvents, with heat-stable enzymes and reduced oxygen requirements (21, 48).

A wide range of studies have dealt with biotransformation, biodegradation, and bioremediation of petroleum hydrocarbons (30, 31, 48, 415, 490, 523), and interest in exploiting petroleum-degrading organisms for environmental clean-up has become central to petroleum microbiology (29). A common theme of early reviews focused on the examination of factors, including nutrients, physical state of the oil, oxygen, temperature, salinity, and pressure, influencing petroleum biodegradation rates, with a view to developing environmental applications (29). Metabolic studies were implemented on the aerobic pathways for alkane, cycloalkane, and aromatic and polycyclic aromatic hydrocarbon (PAH) biodegradation (103, 104, 294, 301, 479, 572, 596, 656), for transformations of nitrogen and sulfur compounds (55, 74, 75, 299, 352, 417), and, more recently, the microbial mechanisms of anaerobic hydrocarbon catabolism (203, 243, 250, 581, 390, 482, 664).

Most significantly, through the developments and applications of molecular techniques, our understanding of the processes of hydrocarbon catabolism has advanced substantially, and many novel catalytic mechanisms have been characterized. A molecular approach is also contributing to a more detailed characterization of bacterial membrane structure. We are learning a great deal about cellular and other physiological adaptations to the presence of hydrocarbons, as well as the

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biochemical mechanisms involved in hydrocarbon accession and uptake (143, 251, 566). The use of genetically engineered microbes for bioremediation has also been considered (210).

The vast range of substrates and metabolites present in hydrocarbon-impacted soils surely provides an environment for the development of a quite complex microbial community. Culture-based methods and culture-independent methods are being developed and implemented to improve our understanding of these microbial communities. Isolating and identifying microorganisms responsible for hydrocarbon transformations have long been recognized as important from a fundamental and applied viewpoint, and lists of hydrocarbon-degrading organisms (bacteria, yeasts, fungi, and algae) are available (30, 33, 366, 522). Leahy and Colwell (366) discussed colony hybridization and dot blot assays in their review and cited molecular tools as revolutionary for describing microbial communities. Magot et al. (398) recently reviewed the current state of knowledge of microorganisms from petroleum reservoirs, including mesophilic and thermophilic sulfate-reducing bacteria, methanogens, mesophilic and thermophilic fermentative bacteria, and iron-reducing bacteria. Again, molecular tools were called upon to provide more detailed community characterizations. These and related studies should provide us with new information on the long-term ecological effects of petroleum pollution and give us directions, for example, regarding the development of new remedial approaches and methods to control some of the deleterious microbial activities occurring during petroleum production.

Current applied research on petroleum microbiology encompasses oil spill remediation (490, 492, 598), fermentor- and wetland-based hydrocarbon treatment (212, 281, 336, 530, 569), biofiltration of volatile hydrocarbons (176), microbial enhanced oil recovery (42, 153), oil and fuel upgrading through desulfurization (417, 554) and denitrogenation (55), coal processing (102), fine-chemical production (412, 415), and microbial community-based site assessment (394). The roles and practical applications of chemical and biological surfactants have been widely reviewed (260, 454, 529, 643).

Oil spill treatment on shorelines and problems associated with open-ocean remediation have been discussed through case histories in numerous reviews (30, 31, 44, 489, 599). Other practical applications include land- and reactor-based refinery waste treatment, in situ tanker ballast cleaning, and subsurface remediation (31, 44).

Heavy crude oil recovery, facilitated by microorganisms, was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery (153). As of 1998, only one productive microbial enhanced oil recovery project was being carried out in the United States (613), although in situ biosurfactant and biopolymer applications continue to garner interest (42).

A limited number of studies have been carried out on biological methods of removing heavy metals such as nickel and vanadium from petroleum distillate fractions, coal-derived liquid shale, bitumens, tars, and synthetic fuels (188, 429, 487, 488, 673). In one approach, cytochrome *c* reductase and chloroperoxidase enzymes have shown potential for metal removal from petroleum fractions. However, further characterization on the biochemical mechanisms and bioprocessing issues involved in heavy metal removal are required in order to develop a reliable biological process.

Bacteria with selected petroleum-metabolizing enzymes amenable to being linked to electronic interfaces are being engineered and developed as biosensors (142). These systems have applications in monitoring environmental contaminant concentrations and toxicities during implementation of remedial processes and also have potential applications in control of environmental processes.

This review deals with developments in our knowledge of petroleum microbiology and in the application of microorganisms in oil bioprocesses and as biosensors. Advances in our understanding of microbial catabolism are presented, including an evaluation of the biochemical mechanisms that control microbial responses to hydrocarbon substrates. These aspects include changes in membrane architecture, active uptake and efflux of hydrocarbons and chemotaxis, and the potential for coordinate control of some of these systems to allow metabolism to take place. Developments in oil bioprocessing focus on transformation of wastes and on the production and upgrading of petroleum and petrochemicals, with emphasis placed on maximizing the rates and extents of microbial growth, hydrocarbon accession, and transformation. Sections dealing with desulfurization and fine-chemical synthesis additionally illustrate the potential benefits of recombinant strains containing enzymes with enhanced activity and/or altered substrate specificity. The possible use of biosensors for online monitoring of pollutants is also addressed.

METABOLISM

Aerobic Alkane Metabolism

Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source. The fundamental aspects of *n*-alkane metabolism and the genes involved have been known for some time. While significant gains have been made in our understanding of the processes involved, the specifics of individual systems and the diversity of systems are yet to be fully described. This section will highlight the recently discovered variability in both the regulation and clustering of alkane degradation genes between species as well as the realization that a single strain may carry multiple genes that code for different enzymes carrying out similar functions. A few rare metabolic pathways will also be discussed.

From a regulatory genetic standpoint, the most extensively characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (formerly *Pseudomonas oleovorans*) (626, 627). Here, a membrane-bound monooxygenase and soluble rubredoxin and rubredoxin reductase serve to shunt electrons through NADH to the hydroxylase for conversion of an alkane into an alcohol. The alcohol can be further oxidized to an aldehyde and acid prior to proceeding into the β-oxidation and tricarboxylic acid cycles. Recently, van Beilen et al. (626, 627) studied the OCT plasmid, while Canosa et al. (98) and Panake et al. (470) examined expression of the AlkS regulator, and Yuste et al. (683, 684) studied the catabolite repression system.

A model for alkane metabolism, including the locations of the Alk proteins and regulation of the *alk* genes, is shown in

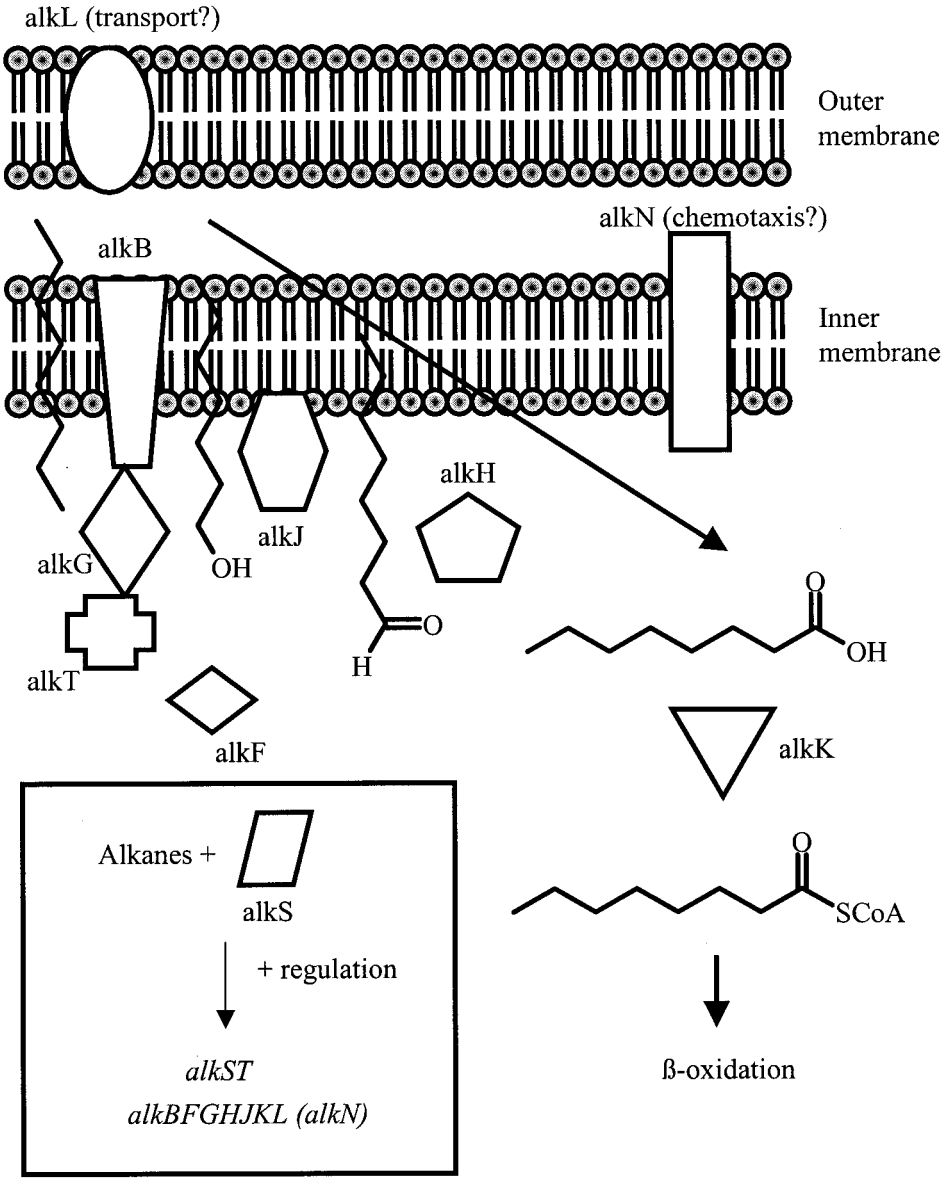

FIG. 1. Schematic of alkane degradation in gram-negative bacteria, showing the locations and functions of the *alk* gene products. The products include AlkB (alkane hydroxylase), AlkF and AlkG (rubredoxins), AlkH (aldehyde dehydrogenase), AlkJ (alcohol dehydrogenase), AlkK (acyl-CoA synthetase), AlkL (outer membrane protein that may be involved in uptake), AlkN (a methyl-accepting transducer protein that may be involved in chemotaxis), AlkT (rubredoxin reductase), and AlkS (positive regulator of the *alkBFGHIJKL* operon and *alkST* genes).

Fig. 1 (627). Here, the *alkBFGHJKL* operon encodes the enzymes necessary for converting alkanes into acetyl-coenzyme A (CoA), while *alkST* encode a rubredoxin reductase (AlkT) and the positive regulator for the *alkBFGHJKL* operon (AlkS). These two operons are located end to end, separated by 9.7 kb of DNA, within which lies *alkN*, a gene coding for a methyl-accepting transducer protein that may be involved in alkane chemotaxis. Note that of all the genes described, the function of *alkL* remains unknown, although it is suspected to be involved in transport. Comparative analysis of insertion sequences in *P. putida* P1 and the previous observation that the G+C content of the *alk* genes is lower than that of both the host strain and the OCT plasmid suggest that the genes are part of an integrated mobile element. Two other plasmid systems have been partially characterized: the OCT plasmid in *Pseudomonas maltophilia* has an *alkA* gene distinct from that of *P. putida* (374), and the unique pDEC plasmid in *Pseudomonas* sp. strain C12B (347).

As other strains are characterized, it appears that the clustering and regulation of alkane degradation genes varies among the bacteria. *Burkholderia cepacia* has an *alkB* gene that is not linked to other alkane degradation genes as it is in *P. putida* (408). The *PalkB* promoter in this organism is downregulated by catabolite repression more strongly than in *P. putida* GPO1 (683). Other differences include the repression of alkane degradation by citrate and the maintenance of repres-

sion during stationary phase in *B. cepacia*, two phenomena not observed in *P. putida* GPO1. In *Acinetobacter* sp. strain ADP1, *alkM*, the terminal alkane hydroxylase-encoding gene, is regulated by *alkR*, which shows no similarity to the LuxR-UhpA-like *alkS* regulator in *P. putida*. In addition, the genes in *Acinetobacter* sp. strain ADP1 are not found in a large operon or on a plasmid. Indeed, the genes are 396 kb from *rubA* and *rubB*, which encode rubredoxin and rubredoxin reductase (213, 505).

The *alkM*, *rubA*, and *rubB* genes in *Acinetobacter* sp. strain M1 are homologous to those in *Acinetobacter* sp. strain ADP1. Interestingly, two alkane hydroxylase complexes (*alkM*a and *alkM*b) whose expression is controlled by *n*-alkane chain length are present in this strain. Conversely, the rubredoxin and rubredoxin reductase are constitutively expressed. Hydropathy plots of AlkMa and AlkMb suggest that the proteins are similar to AlkB in *P. putida* in that they are membrane bound. AlkMa appears to be similar to AlkM of *Acinetobacter* sp. strain ADP1. The first of two transcriptional regulators in *Acinetobacter* sp. strain M1 (AlkRa) is related to AraC-XylS type regulators, which includes that of *Acinetobacter* sp. strain ADP1. The second regulator (AlkRb) is similar to OruR of *P. aeruginosa*. The two regulators are induced by different *n*-alkanes in this strain. *alkMa* responds to solid, long-chain alkanes ($>C_{22}$), while *alkMb* responds to liquid alkanes (C_{16} to C_{22}). Unlike the case in *P. putida*, neither acetate nor hexadecanol induces *alkMa* and *alkMb* (602).

The presence of multiple alkane hydroxylase genes in a single strain does not appear to be a unique phenomenon. Two distinct monooxygenases, a Cu-containing monooxygenase and an integral-membrane, binuclear-iron monooxygenase similar to that of *P. putida* GMo1 have been described in *Nocardiodes* sp. strain CF8 (233). While the Cu-containing monooxygenase is expressed in response to a wide range of alkanes, only those with more than six carbons induce the binuclear-iron monooxygenase. Once again, the genes encoding alkane metabolism in *Acinetobacter* sp. strain M1 and *Nocardiodes* sp. strain CF8 are not clustered together as in the OCT plasmid (275, 602). Other enzymes involved in *Acinetobacter* sp. strain M1 alkane metabolism have been characterized. Ishige et al. (275) isolated a soluble long-chain NAD^+-dependent aldehyde dehydrogenase whose activity increased with increasing aldehyde chain length (tetradecanal preferred) that is encoded by the chromosomal *ald1* gene. This enzyme plays a role in both alkane degradation and biosynthesis, depending on the conditions. The NAD^+-dependent aldehyde dehydrogenase in strain HD1 is also reported to prefer long-chain aldehydes (462). A thermostable $NADP^+$-dependent medium-chain alcohol dehydrogenase, encoded by *alrA*, has also been isolated but is not believed to participate in the main alkane oxidation pathway due to its cytosolic location and greater activity towards medium-chain alcohols (603).

Despite the importance of alkane degradation systems, little information is available for pathways other than the aerobic monooxygenase-mediated pathway found on the OCT plasmid. Evidence for the Finnerty pathway, where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate, has been described for *Acinetobacter* sp. strain M1 (397, 534). The dioxygenase requires molecular oxygen to catalyze the oxidation of *n*-alkanes (C_{10} to C_{30}) and alkenes (C_{12} to C_{20}) without the production of oxygen radicals. A flavin adenine dinucleotide chromophore was detected, and the enzyme is thought to contain Cu^{2+}. Unlike the case for the 1-monooxygenase in *P. putida*, rubredoxin and NAD(P)H are not required.

Another novel metabolic pathway has been observed in a *Rhodococcus* mutant (338). In this case, aliphatics are *cis*-desaturated, producing products with double bonds mainly at the ninth carbon from the terminal methyl group. It is postulated that a coenzyme A-independent *cis*-desaturase may be involved in this activity. Dutta and Harayama (159) recently noted that the degradation of the long side chains of *n*-alkylbenzenes and *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326 proceeds mainly by β-oxidation (Fig. 2). However, minor products suggest the possibility of other degradative routes. For example, 4-cyclohexylbutanoic acid was metabolized through 4-cyclohexyl-2-butenoic acid (β-oxidation) and other intermediates not believed to be formed by β-oxidation (4-cyclohexyl-3-butenoic acid and cyclohexylcarboxylic acid).

In the above cases, there is much work to be done with respect to describing both the genetic systems and the enzymes involved. Even more challenging will be answering questions such as what role these pathways play in environmental remediation, how the different approaches to alkane metabolism evolve and how are they related, and how well-characterized and novel metabolic pathways can be applied in fine-chemical synthesis.

Aerobic PAH Metabolism

A great deal of work has been carried out in trying to rationalize the persistence of PAH in the environment. As more studies are carried out, it is becoming increasingly evident that a vast array of microbial species (bacteria, fungi, algae, and cyanobacteria) have a diversity of tools to use both low- (three rings or fewer) and high-molecular-weight (four or more rings) PAHs such as naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, and chrysene as sole carbon and energy sources. While no strains have yet been found to utilize PAHs with more than four rings, such as benzo[*a*]pyrene as a sole carbon and energy source, cometabolic transformations have been characterized (for reviews, see references 103, 104, 294 301, 572, 596, and 597).

The low water solubility and high sorbtion capacity of PAHs are often found to greatly influence biodegradation, but other factors, including production of toxic or dead-end metabolites, metabolite repression, the presence of preferred substrates, and the lack of cometabolic or inducer substrates, must be considered when PAH persistence is evident (433, 295). Understanding how these factors affect the transformation of and determining any given PAH is difficult; understanding the processes in natural environments when mixtures of PAHs and their myriad metabolites are present is more difficult, especially as the majority of work has focused on a narrow selection of species. Indeed, the cited reviews generally conclude by calling for more study into the regulation of PAH biodegradation, biodegradation of PAH mixtures, and interactions within microbial consortia.

Until recently, the majority of information on the genetics of PAH metabolism has come from studying naphthalene catabolic plasmids such as NAH7 from *Pseudomonas putida* strain

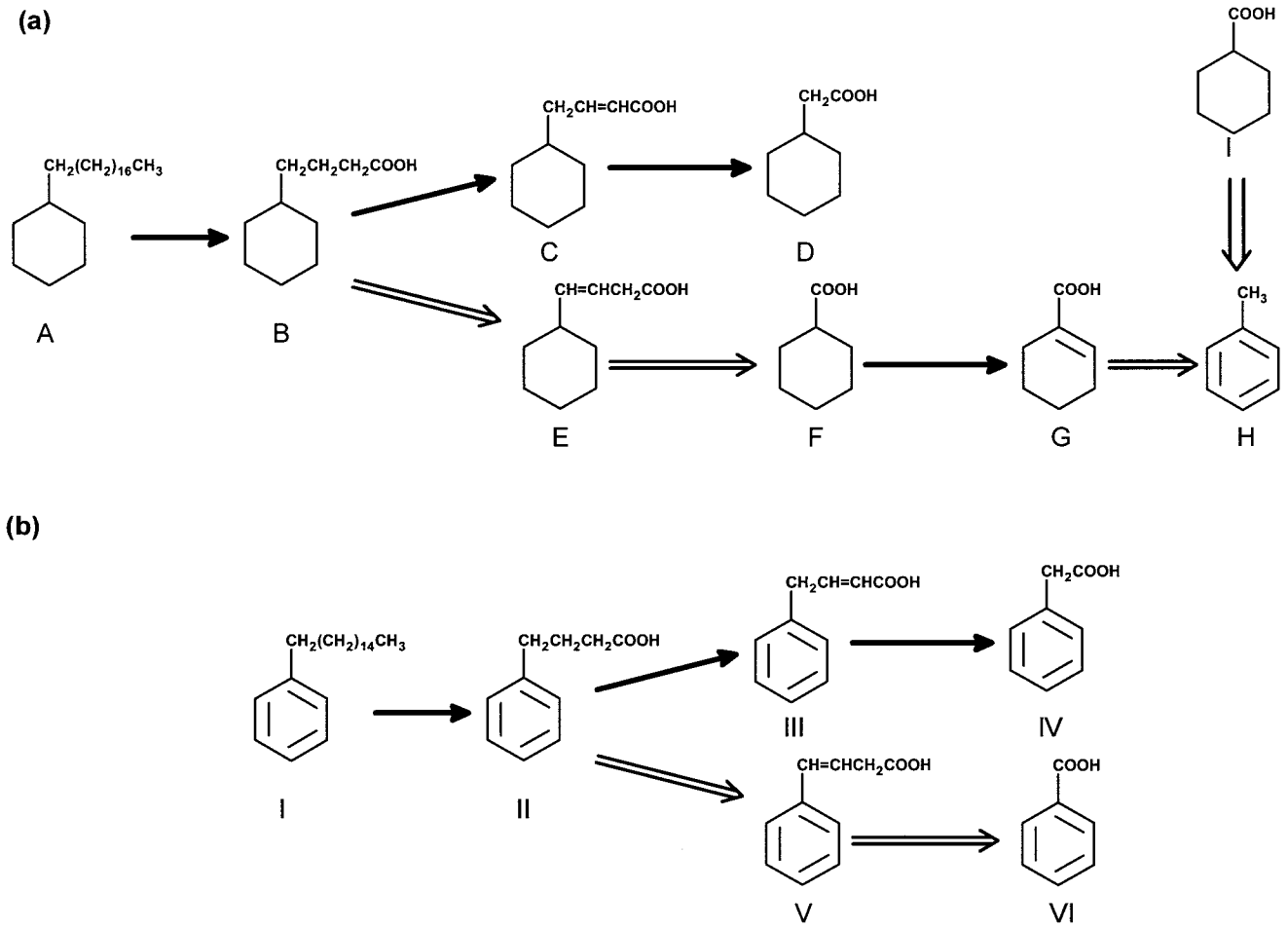

FIG. 2. Proposed metabolic pathway illustrating biodegradation of an *n*-alkylcyclohexane (a) and an *n*-alkylbenzene (b) by an *Alcanivorax* sp. strain MBIC4326 (adapted from reference 159). The major metabolic route of β-oxidation is shown with bold arrows, while minor routes are indicated with open arrows and a novel metabolic route by large open arrows. Pathway a: A, *n*-octadecylcyclohexane; B, 4-cyclohexabutanoic acid; C, 4-cyclohexyl-2-butanoic acid; D, cyclohexane acetic acid; E, 4-cyclohexyl-2-butenoic acid; F, cyclohexane carboxylic acid; G, 1-cyclohexene-1-carboxylic acid; H, benzoic acid; I, 3-cyclohexene-1-carboxylic acid. Pathway b: I, *n*-hexadecylbenzene; II, 4-phenylbutanoic acid; III, 4-phenylbutenoic acid; IV, phenylacetic acid; V, 4-phenylbutenoic acid; VI, benzoic acid.

G7. In this well-characterized system, the first operon (*nah AaAbAcAdBFCED*) encodes the pathway for naphthalene conversion to salicylate (upper pathway), and the second (*nah GTHINLOMKJ*) codes for the conversion of salicylate via catechol *meta*-cleavage to acetaldehyde and pyruvate (lower pathway) (164, 485, 568, 679). The regulator for both operons is encoded by a third operon containing *nahR*, which is induced by salicylate (547). Here, molecular oxygen is introduced into the aromatic nucleus via naphthalene dioxygenase, a multicomponent nonheme iron oxygenase enzyme system consisting of a reductase, a putative Rieske [2Fe-2S] iron sulfur center in a ferredoxin, and an iron-sulfur flavoprotein. The initial reaction results in the formation of *cis*-naphthalene dihydrodiol, which is subsequently converted to salicylate and then to tricarboxylic acid intermediates (for more detail, see references 104, 220, and 679). As will be discussed below, naphthalene dioxygenase is now known to be a versatile enzyme, able to catalyze a wide variety of reactions. Molecular and biochemical evidence that the naphthalene plasmid degradative enzyme system could mineralize other PAHs, such as phenanthrene and anthracene, was first provided by two research groups in 1993 (423, 540).

As more PAH-degrading bacteria were isolated and characterized, and as molecular methods to study microbial communities developed, the diversity of PAH metabolic genes was discovered. Examples of bacteria with unknown, nonhomologous genes to the naphthalene NAH7-like catabolic plasmids have been reported recently (318, 528). At the same time, a variety of new isofunctional gene sequences have been reported in different bacterial species, most notably in *Nocardia*, *Rhodococcus*, and *Mycobacterium* spp., some of which are capable of using high-molecular-weight PAHs such as pyrene as carbon and energy sources (Table 1).

High levels (≈90%) of homology and a conserved gene arrangement are observed in the *nah*, *ndo*, *pah*, and *dox* sequences (63, 64, 147, 333, 355, 601). In fact, it has been proposed that the *dox* plasmid, which encodes a dibenzothiophene (DBT) metabolic pathway analogous to the naphthalene catabolic pathway, may in fact be a naphthalene catabolic plasmid (163). High homology, however, does not necessarily translate

TABLE 1. Chromosomally and plasmid-encoded polycyclic aromatic hydrocarbon degradation gene clusters, illustrating the diversity of operon organization

Strain	Location	Substrate	Gene	Encoded protein or function	Reference
Pseudomonas putida strains	Plasmid	Naphthalene (upper pathway)	*nahAa*	Reductase	568
			nahAb	Ferredoxin	679
			nahAc	Iron sulfur protein large subunit	485
			nahAd	Iron sulfur protein small subunit	
			nahB	*cis*-Naphthalene dihydrodiol dehydrogenase	
			nahF	Salicyaldehyde dehydrogenase	
			nahC	1,2-Dihydroxynaphthalene oxygenase	
			nahE	2-Hydroxybenzalpyruvate aldolase	
			nahD	2-Hydroxychromene-2-carboxylate isomerase	
		Salicylate (lower pathway)	*nahG*	Salicylate hydroxylase	
			nahT	Chloroplast-type ferredoxin	
			nahH	Catechol oxygenase	
			nahI	2-Hydroxymuconic semialdehyde dehydrogenase	
			nahN	2-Hydroxymuconic semialdehyde dehydrogenase	
			nahL	2-Oxo-4-pentenoate hydratase	
			nahO	4-Hydroxy-2-oxovalerate aldolase	
			nahM	Acetaldehyde dehydrogenase	
			nahK	4-Oxalocrotonate decarboxylase	
			nahJ	2-Hydroxymuconate tautomerase	
		Regulator for both operons	*nahR*	Induced by salicylate	547
Pseudomonas putida NCIB9816	Plasmid	Naphthalene	*ndoA*	Naphthalene-dioxygenase genes (these 3 genes correspond to NahAb,-c, and-d listed above)	355
			ndoB		
			ndoC		
Pseudomonas sp. strain C18	Plasmid	Dibenzothiophene	*doxA*	Naphthalene dioxygenase	148
		Naphthalene phenanthrene	*doxB*	DoxA, -B, -D correspond to NahAb, -c, and-d listed above	
			doxD		
			doxE	*cis*-Naphthalene dihydrodiol dioxygenase	
			doxF	Salicylaldehyde dehydrogenase	
			doxG	1,2-Dihydroxynaphthalene dioxygenase	
			doxH	Isomerase (interchangeable with *doxJ*?)	
			doxI	Hydratase-aldolase	
			doxJ	Isomerase	
Pseudomonas sp. strain U2	Plasmid	Naphthalene	*nagAa*	Ferredoxin reductase	205
			nagG	Subunit of salicylate 5-hydroxylase with Rieske-type iron-sulfur centre	
			nagH	Subunit of salicylate 5-hydroxylase	
			nagAb	Ferredoxin	
			nagAc	Large dioxygenase subunit	
			nagAd	Small dioxygenase subunit	
			nagB	Naphthalene *cis*-dihydrodiol dehydrogenase	
			nagF	Salicylaldehyde dehydrogenase	
Burkholderia sp. strain RP007	Plasmid	Naphthalene phenanthrene	*phnR*	Regulatory	364
			phnS	Regulatory	
			phnF	Aldehyde dehydrogenase	
			phnE	Hydratase-aldolase	
			phnC	Extradiol dioxygenase	
			phnD	Isomerase	

			phnAc	Large dioxygenase subunit (Rieske-type [2Fe-2S]	
			phnAd	Small dioxygenase subunit	
			phnB	Dihydrodiol dehydrogenase	
Pseudomonas putida OUS82	Chromosome	Naphthalene Phenanthrene A variety of homo-hetero-, and monocyclics converted to phenols	*pahAa*	Ferredoxin reductase	333
			pahAb	Ferridoxin	601
			pahAc	Large subunit of iron-sulfur protein	
			pahAd	Small subunit of iron-sulfur protein	
			pahB	*cis*-Dihydrodiol dehydrogenase	
			pahC	Dioxygenase	
			pahD	Isomerase	
			pahE	Hydratase-aldolase	
			pahF	Dehydrogenase	
Pseudomonas stutzeri AN10	Chromosome	Naphthalene 2-Methylnaphthalene	*nahG*	Salicylate 1-hydroxylase	63
			nahW	Salicylate 1-hydroxylase (outside *meta*-cleavage transcriptional unit)	
Nocardiodes sp. strain KP7	Chromosome	Phenanthrene	*phdA*	Alpha subunit of dioxygenase	533
			phdB	Beta subunit of dioxygenase	
			phdC	Ferredoxin	
			phdD	Ferredoxin reductase	
			phdK	2-Carboxybenzaldehyde dehydrogenase	
Rhodococcus sp. strain 124	Chromosome	Naphthalene Toluene Indene	*nidA*	Naphthalene-inducible dioxygenase system	615
			nidB	Dioxygenase small subunit	
			nidC	*cis*-Dihydrodiol dehydrogenase	
			nidD	Putative aldolase	
Mycobacterium sp. strain PYR-1	Chromosome	Anthracene, Phenanthrene Fluoranthene Pyrene, benzo [a]pyrene, 1-nitropyrene	*nidD*	Aldehyde dehydrogenase	318
			nidB	Small subunit of dioxygenase	
			nidA	Large subunit of dioxygenase	
Sphingomonas paucimobilis var. EPA505		Phenanthrene Anthracene, benzo[b]fluoranthene Naphthalene Fluroanthene, pyrene Intermediate catabolites	*pbhA*	Ring fission dioxygenase	587
			pbhB	Rieske-type ferridoxin subunit of multicomponent dioxygenase	
			pbhC	Hydratase-aldolase	
			pbhD	Pyruvate phosphate dikinase	

to similar substrate specificities, as Yang et al. (677) found that *P. putida* NCIB 9816 clones were able to produce metabolites from naphthalene, fluorine, and phenanthrene, while only naphthalene metabolites were detected from a homologous NAH7 clone.

In addition, with respect to PAH metabolism, novel gene sequences and gene orders have been observed in a variety of strains, including *Burkholderia* sp. strain RP007, *phnFECDAc AdB* (364); *Pseudomonas* sp. strain U2, *nagAaGHAbAcAdBF* (205); *Rhodococcus* sp. strain I24, *nidABCD* (615); *Mycobacterium* sp. strain PYR1, *nidDBA* (318); and *Nocardiodes* sp. strain KP7, *phdABCD* (542). Sequence diversity, and the fact that naphthalene catabolic genes have now been found on the chromosome as well as on plasmids indicate that lateral gene transfer and genetic recombination may have played an important role in the development of these versatile metabolic pathways (63, 64, 205, 364, 542). For example, the *phn* locus has similarities to both *nah* and *bph* genes in *Burkholderia* sp. strain RP007 (364), while the chromosomally encoded *nah* upper and lower pathways in *Pseudomonas stutzeri* AN10 appear to have been recruited from other organisms and recombined. In fact, two entire *nah* upper pathways may exist in this strain (63, 64).

Thus, not only are new gene sequences being found for PAH metabolism, but strains possessing multiple genes for similar enzymes are being detected. Ferrero et al. (189) recently showed, while studying *Pseudomonas* spp. isolated from the western Mediterranean, that single strains can have two distinct *nahAc*-like genes as well as other genes of the upper *nah* pathway. With respect to the lower pathway, Bosch et al. (63) found two distinct genes for salicylate 1-hydroxylase, the flavoprotein monooxygenase that converts salicylate to catechol, on the chromosome of *P. stutzeri* AN10. While the *nahG* gene was found in the *meta*-cleavage pathway transcriptional unit, the novel *nahW* was found close to but outside of this unit. Both are induced upon exposure to salicylate and have broad substrate specificities, but *nahW* is missing the conserved flavin adenine dinucleotidebinding site (GxGxxG) normally found in these hydroxylases. This is the first example of two isofunctional salicylate hydroxylases in one strain, and it will be interesting to discover if the combination of genes from various catabolic routes is a widespread phenomenon.

This type of metabolic expansionism is exemplified by *Sphingomonas yanoikuyae* B1, which has recruited, modified, and reorganized genes to obtain catabolic pathways for naphthalene, phenanthrene, anthracene, biphenyl, toluene, and *m*- and *p*-xylene. In this case, *nah*, *bph*, and *xyl* genes are present but are not arranged in three distinct operons (215, 330, 692). Indeed, this gene clustering may be typical of *Sphingomonas* spp. capable of degrading aromatic compounds. Romine et al. (519, 520) sequenced the pNL1 (≈184 kb) plasmid of *Sphingomonas aromaticivorans* F199, which is capable of degrading toluene, xylenes, salicylate, biphenyl, dibenzothiophene, flourene, and benzoate. In this plasmid, at least 13 gene clusters are predicted to encode all of the necessary enzymes. In addition, seven three-component oxygenases with components spread over six gene clusters have been predicted.

Beyond the genes known to participate directly in PAH metabolism, genes that may provide important support functions are being described. *Sphingomonas paucimobilis* var. EPA500, a strain able to use fluoranthene, naphthalene, and phenanthrene as sole carbon and energy sources, has *pbhD*, a gene encoding pyruvate phosphate dikinase homologous to *ppdK* that is known to be involved in glucose uptake in prokaryotes and plants. If *pbhD* is disrupted, fluoranthene metabolism is interrupted. While the gene function is not clear, it is possible that it is involved in the uptake of fluoranthene catabolites that leak from the cell (587). Another example is the *katG* gene in *Mycobacterium* sp. strain PYR-1, which encodes an 81-kDa catalase-peroxidase induced upon exposure to pyrene (651). This enzyme may protect the dioxygenase from oxidative inactivation by exogenous oxidation or by removing H_2O_2 generated endogenously during PAH metabolism (375, 426, 651). Grimm and Harwood (226, 227) recently found *nahY* on the NAH7 catabolic plasmid of *P. putida* G7, which encodes a membrane protein that may be a chemoreceptor for naphthalene or naphthalene metabolites.

In order to move towards a better understanding of the diversity of PAH metabolism in the ecosystem, research should be directed towards genera other than mesophilic pseudomonads. This will allow a variety of research questions to be addressed: what impact different genera have on PAH metabolism in the evironment; what and how pathways should be encouraged in active bioremedation systems; and what relationship exists between ecosystem properties and PAH metabolism.

To start, synergistic and antagonistic interactions between PAHs of both high and low molecular weights are being investigated. For example, Molina et al. (433) observed that, for both a mixed culture and *Mycobacterium* sp. strain M1, cross-acclimation occurred between phenanthrene and pyrene metabolism in that pyrene-grown cells did not require new protein synthesis to degrade phenanthrene. On the other hand, neither naphthalene nor anthracene resulted in induction or inhibition of pyrene mineralization. Samanta et al. (537) found that phenanthrene mineralization increased in two strains when fluorine, fluoranthene, and pyrene mixtures were added, while mineralization was not affected in two other strains. In this case, a consortium of the four strains did not enhance phenanthrene mineralization, as has been observed in other studies with defined bacterial and bacterial-fungal consortia (61, 67, 101, 616).

Inhibition may also occur, presumably due to competition for enzymes involved in oxidation or transport, accumulation of by-products resulting in cytotoxicity, and blockage of enzyme induction (66, 295, 590, 564). Determining which mechanism is important in any given situation can be complicated by the presence of metabolites from the different PAHs. The pyrene metabolite *cis*-4,5-dihydro-4,5-dihydroxypyrene inhibited phenathrene metablism in *Pseudomonas saccharophila* strain P15 and *Sphingomonas yanoikuyae* R1 but had little effect on *Pseudomonas stutzeri* P16 and *Bacillus cereus* P21 (313). In addition, the above metabolite and its oxidation product, pyrene-4,5-dione, inhibited benzo[*a*]pyrene mineralization in the sensitive strains. In a follow-up study, the strains were found to form the dead-end product fluoranthene-2,3-dione as a cometabolic product of flouranthene when grown on phenanthrene. Phenanthrene removal was inhibited by this metabolite in *Sphingomonas* sp. strain R1 but not in the three other strains studied. Mineralization of benz[*a*]anthracene, benzo[*a*]pyrene, and chrysene was also inhibited in R1, while only benzo-

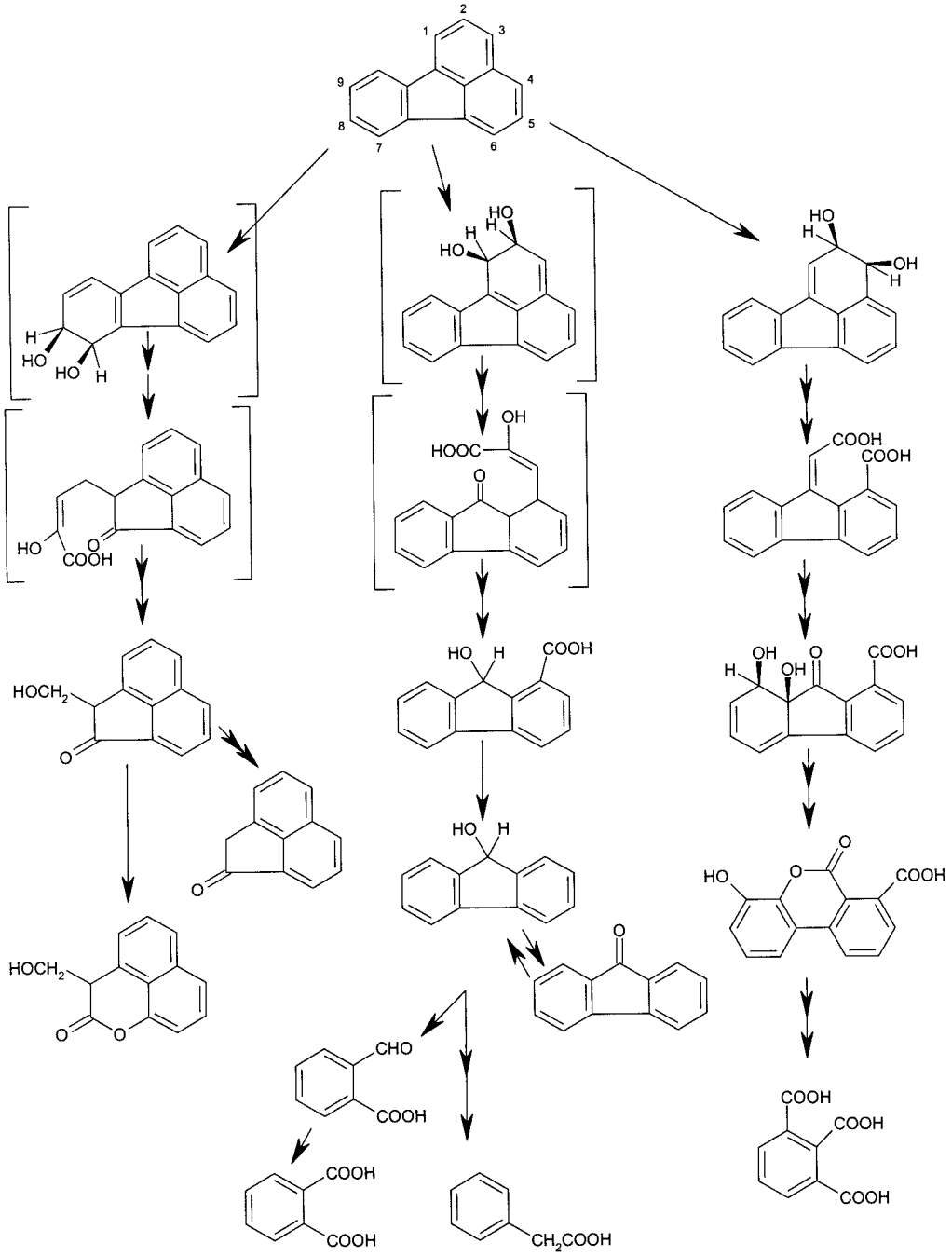

FIG. 3. Bacterial fluoranthene biodegradation pathways, illustrating microbial metabolic diversity with respect to high-molecular-weight PAHs. Intermediates in brackets have not yet been identified.

[*a*]pyrene metabolism in P15 was affected. Cytotoxicity was partly responsible for the observed inhibition (314). Thus, depending on the strains, transformation products from one PAH may affect the removal of other PAHs (295, 112). Overall, induction effects in complex mixtures may be as important as diauxic effects (49, 304, 305, 418).

Understanding how a metabolite may interact with a specific receptor or enzyme requires knowledge of what metabolites are formed and how persistent they are in the environment. Indeed, the number of known metabolites from both low- and high-molecular-weight PAHs is increasing as more researchers apply techniques such as high-resolution gas chromatography-mass spectroscopy and nuclear magnetic resonance in their studies. Recent studies with members of the mycobacteria, ubiquitous soil microorganisms with versatile metabolic abilities, illustrate the diversity of PAH metabolic pathways.

For example, Grund et al. (230) noted that *Rhodococcus* sp. strain B4, whose naphthalene metabolic pathway was not in-

duced by salicylate, the normal inducer of the NAH7 pathway, oxidized salicylate to gentisate rather than catechol. More recently, Dean-Ross et al. (144) described a *Rhodococcus* sp. that metabolizes anthracene to 1,2-dihydroxyanthracene and then to either 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid or 6,7-benzocoumarin. The second product is from the *meta*-cleavage pathway found in both gram-positive and gram-negative bacteria, while the first product is from a novel *ortho*-pathway, to date only identified in gram-positives (22, 437, 641). In gram-negatives, novel metabolic pathways for low-molecular-weight PAHs, such as phenanthrene and fluorene, have been recently described as well (100, 537).

The number of strains known to utilize four-ring PAHs as sole carbon and energy sources, even in the absence of cofactors or surfactants, and those known to cometabolize PAHs with more than four rings has increased greatly in the last 10 years. Along with this, a myriad of metabolic pathways have been proposed, as documented by Kanaly and Harayama (301) for a variety of high-molecular-weight PAHs in bacteria, and by Juhasz and Naidu (294), who focused on microbial metabolism of benzo[*a*]pyrene. In the short time since these reviews appeared, more examples of novel metabolic pathways and cooxidation products have been described. For example, Rehmann et al. (507) outlined a new pathway for fluoranthene metabolism in *Mycobacterium* sp. strain KR20, whereby initial dioxygenation commences at the 2,3 position (Fig. 3). Kazunga et al. (314) identified fluoranthene-2,3-dione and fluroanthene-1,5-dione as dead-end metabolites from fluoranthene during growth on phenanthrene in *Pseudomonas saccharophila* strain P15, *Sphingomonas yanoikuyae* strain R1, *Pseudomonas stutzeri* P16, and *Bacillus cereus* strain P2. These metabolites are not likely to be intermediates of fluoranthene metabolism, but instead are probably autooxidation products of the corresponding *o*-dihydroxy metabolites.

It is becoming evident that many strains employ monoxygenases or both monooxygenases and diooxygenases for the metabolism of single-ring PAHs (20, 437, 614, 641). In addition, classic dioxygenase enzymes such as the multicomponent naphthalene dioxygenase can catalyze monohydroxylation, dihydroxylation, desaturation, O- and N-dealkylation, and sulfoxidation reactions against a wide variety of monocyclic and heterocyclic compounds (217, 369, 509, 553). Site-directed mutagenesis of naphthalene dioxygenase indicates that slight changes in amino acid sequence can have profound effects on reaction regio- and stereospecificity (681). Questions related to enzyme functionality and the evolution of similar naphthalene dioxygenases in different genera (e.g., *Pseudomonas* and *Rhodococcus*) will be answered as more enzymes are purified and characterized (93, 237, 310, 368, 414, 361, 472, 591, 592).

Overall, the broad PAH-degrading capabilities in many strains may be attributed to relaxed initial enzyme specificity for PAHs (low and high molecular weight and methyl substituted), the presence of multiple oxygenases, and the presence of multiple metabolic pathways or multiple genes for isofunctional pathways (83, 112, 160, 249, 220, 330, 396, 399, 418, 437, 519, 520, 532, 641, 677). Finally, the presence of both alkane and aromatic compound-degrading genes within single strains appears to be common (120, 301, 576, 578, 641, 662).

How these various metabolic routes are controlled at the genetic level and how they compete for a substrate is still a major question. This is especially evident when novel dead-end metabolites, such as the methoxylated 1-methoxy-2-hydroxyanthracene from anthracene metabolism (641) and the dicarboxylic acid 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid from pyrene metabolism (437), are detected with strains simultaneously employing multiple degradative routes for a single substrate. This is also the case in strains that have degradative pathways for multiple aromatic substrates (588, 519). For example, in *Sphingomonas aromaticivorans* strain F199, induction studies have indicated that naphthalene and toluene mineralization may be higher in the presence of both substrates, as greater gene expression can be achieved (519).

Anaerobic Hydrocarbon Metabolism

Anaerobic metabolism is a vital process with respect to petroleum hydrocarbon biodegradation and bioremediation and, given the unique biochemistry now being uncovered, is also vital with respect to biomimetic catalyst development. Currently, we are in a period of rapid expansion with quality, convention-shattering work being released at an exciting pace. This is evidenced by the number of reviews being published in the area after 10 years of accelerated discovery (203, 243, 250, 271, 390, 482, 581, 664). Given the scope of the current reviews, a brief overview of anaerobic hydrocarbon metabolism will be given, with mention of some new advances since Widdel and Rabus published their review in 2001 (664).

Work with microbial consortia in the field, in enrichment cultures, and in microcosms has illustrated that hydrocarbons such as toluene (171, 358), alkylbenzenes including *m*-, *o*-, and *p*-xylene and trimethylbenzenes (39, 111, 235, 481), benzene (90, 312, 521), naphthalene and phenanthrene (50, 124, 421, 686), methylnaphthalene and tetralin (20, 23), $>C_6$ *n*-alkanes (18, 96, 168, 575), branched alkanes (72, 73), and hydrocarbon mixtures (228) can be metabolized under anaerobic conditions. These reactions may take place under Fe(III)-reducing, denitrifying, and sulfate-reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria. Other terminal electron acceptors shown to be used during anaerobic hydrocarbon metabolism include manganese oxides (357, 358), soil humic acids and the humic acid model compound anthraquinone-2,6-disulfonate (105), and fumarate in a fermentative oxidation process (420). Mixed-culture work continues as enhanced bioremediation strategies are tested (17, 530) and new metabolites are described (23, 172, 421, 687).

More recently, the number of pure cultures shown to metabolize various hydrocarbons with different electron acceptors has increased (Table 2). This diverse set of bacteria (no fungi have been studied to date), including members of the α-, β-, γ-, and δ-subclasses of the proteobacteria, form an excellent framework from which to elucidate the underlying biochemical and molecular mechanisms driving anaerobic hydrocarbon metabolism.

Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterizations in the denitrifying bacteria *Azoarcus* sp. strain T, *Thauera aromatica* strain K172, and *Thauera* sp. strain T1 (2, 52, 53, 54, 58, 135, 136, 250, 255, 349, 378, 379). In the proposed pathway, fumarate addition to toluene is mediated by benzylsuccinate synthase to form ben-

TABLE 2. Petroleum hydrocarbon-degrading anaerobic bacteria

Organism	Hydrocarbon(s) used	Reference
Anoxygenic photoheterotrophic bacterium		
Blastochloris sulfoviridis ToP1	Toluene	685
Denitrifying bacteria		
Azoarcus sp. strain EB1	Ethylbenzene	38
Azoarcus sp. strain T	Toluene, *m*-xylene	152
Azoarcus tolulyticus Td15	Toluene, *m*-xylene	204
Azoarcus tolulyticus To14	Toluene	690
Dechloromonas sp. strain JJ	Benzene, toluene	125
Dechloromonas sp. strain RCB	Benzene, toluene	125
Pseudomonas sp. strain NAP-3	Naphthalene	517
Strain EbN1	Ethylbenzene, toluene	495
Strain HdN1	C_{14}–C_{20} alkanes	168
Strain HxN1	C_6–C_8 alkanes	168
Strain M3	Toluene, *m*-xylene	256
Strain mXyN1	Toluene, *m*-xylene	495
Strain OcN1	C_8–C_{12} alkanes	168
Strain PbN1	Ethylbenzene, propylbenzene	495
Strain pCyN1	*p*-Cymene, toluene, *p*-ethyltoluene	238
Strain pCyN2	*p*-Cymene	239
Strain T3	Toluene	256
Strain ToN1	Toluene	495
Thauera aromatica K172	Toluene	16
Thauera aromatica T1	Toluene	181
Vibrio sp. strain NAP-4	Naphthalene	517
Fe(III)-reducing bacteria		
Geobacter grbiciae TACP-2^T	Toluene	123
Geobacter grbiciae TACP-5	Toluene	123
Geobacter metallireducens GS15	Toluene	391
Sulfate-reducing bacteria		
Desulfobacula toluolica To12	Toluene	497
Desulfobacterium cetonicum	Toluene	238
Strain AK-01	C_{13}–C_{18} alkanes	574
Strain Hxd3	C_{12}–C_{20} alkanes, 1-hexadecene	4
Strain mXyS1	Toluene, *m*-xylene, *m*-ethyltoluene, *m*-cymene	238
Strain NaphS2	Naphthalene	208
Strain oXyS1	Toluene *o*-xylene, *o*-ethyltoluene	238
Strain Pnd3	C_{14}–C_{17} alkanes, 1-hexadecene	4
Strain PRTOL1	Toluene	54
Strain TD3	C_6–C_{16} alkanes	531

zylsuccinate. Following this unusual addition reaction, a series of modified β-oxidation reactions are thought to convert benzylsuccinate to benzyl-CoA (52, 53, 58), which is a central intermediate in the anaerobic degradation of aromatic compounds (243).

Benzylsuccinate synthase has been purified from *Azoarcus* sp. strain T and *T. aromatica* strain K172 and is characterized as a $\alpha_2\beta_2\gamma_2$ heterohexamer with a flavin cofactor but no iron-sulfur clusters (54, 378) and represents a new class of glycyl radical-containing enzymes (350). Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase, which activates (*R*)-benzylsuccinate to 2-(*R*)-benzylsuccinyl-CoA, has also been purified from strain *Thauera aromatica* K172 (380).

The genes encoding benzylsuccinate synthase have been cloned and sequenced in *Azoarcus* sp. strain T (2), *T. aromatica* strain K172 (378), and *T. aromatica* strain T1 (135, 136, 137, 378). In strain *T. aromatica* K172, the *bbs* (beta-oxidation of benzylsuccinate) operon contains *bbsDCABE*, with *bbsCAB* encoding the γ, α, and β subunits of benzylsuccinate synthase, a region with significant homology to the *tutFDG* genes in strain T1 (136, 255, 378). The genes encoding the putative activating enzyme (*bssD* and *tutE*) are found upstream and also show homology in the two strains. BssE in K172 may be an ATP-dependent chaperone for assembly or deactivation of benzylsuccinate synthase (255). In contrast to K172 and T1, strain T mineralizes both toluene and *m*-xylene. In this case, expression of the *bssDCABE* operon is required for growth on both substrates (2).

Similar operons may be present in other strains, as the novel benzylsuccinate synthase reaction, catalyzing the addition of fumarate to toluene (110, 181), may also be involved in the metabolism of xylenes (349, 444, 445), alkylnaphthalenes (20, 23), *n*-hexadecane (497), and *n*-dodecane (351). For example, dodecylsuccinic acids were detected from a sulfate-reducing enrichment culture growing on *n*-dodecane (351), and an *n*-hexane-utilizing denitrifying bacterium with a protein similar to BssC has been isolated from the toluene-degrading denitrifying bacteria (664). In addition, the metabolites (1-methylpentyl)succinate and (1-ethylbenzyl)succinate from the anaerobic metabolism of *n*-hexane by a denitrifying strain indicate a C-2

and a C-3 addition of fumarate, analogous to the toluene activation reaction (497). The (1-methylpentyl)succinate is then converted to a CoA thioester prior to rearrangement to (2-methylhexyl)malonyl-CoA and degradation by conventional β-oxidation (666). Thus, it appears that the fate of the alkylsuccinates produced is probably fatty acid metabolism (5, 574, 666).

For ethylbenzene, oxidation under denitrifying conditions appears to commence with a dehydrogenation by ethylbenzene dehydrogenase to produce 1-phenylethanol followed by oxidation to acetophenone (39, 108, 291, 495, 496). Ethylbenzene dehydrogenase has been isolated from both *Azoarcus* sp. strains EB1 (292) and EbN1 (335). In both cases, the enzyme is an αβγ-Mo-Fe-S heterotrimer. Johnson et al. (292) sequenced *ebdA*, encoding the α-subunit containing a molybdopterin-binding domain; *ebdB*, encoding the β-subunit containing several 4Fe-4S binding domains; and *ebdC*, encoding the γ-subunit, a potential membrane anchor subunit. Kniemeyer and Heider (334) isolated the NAD^+-dependent secondary alcohol dehydrogenase (*S*)-1-phenylethanol dehydrogenase, which catalyzes acetophenone formation in *Azoarcus* sp. strain EbN1. Analogous reactions are believed to occur for *n*-propylbenzene (495), while for sulfate-reducing bacteria the metabolic pathway may be similar to that of toluene metabolism, as (1-phenylethyl)succinate has been detected in enrichment cultures (172). It is of interest that *Azoarcus* sp. strain EbN1 also degrades toluene, but via benzylsuccinate (496).

Two- and three-ring PAHs may also be metabolized under anaerobic conditions. For naphthalene, activation proceeds via carboxylation to form 2-naphthoate in sulfate-reducing (208, 438) and denitrifying (517) bacteria. Carboxylation has also been observed for phenanthrene added to a sulfidogenic culture (686). Alkylnaphthalenes appear to be activated by a mechanism similar to that of toluene, as naphthyl-2-methylsuccinate has been detected in sulfate-reducing enrichment cultures exposed to 2-methylnaphthalene (20).

Recently, Annweiler et al. (23) proposed that, with a sulfate-reducing enrichment culture, naphthalene, 2-methylnaphthalene, and tetralin (1,2,3,4-tetrahydronaphthalene) are all degraded, with 2-naphthoic acid being the central intermediate in a pathway analogous to the benzyl-CoA pathway for monoaromatic compounds. Further degradation occurs through saturated compounds with cyclohexane ring structures (also see 687). They have also found that a sulfate-reducing enrichment culture cometabolized benzothiophene when grown with naphthalene. While activity was not very high, perhaps because of inhibition, toxicity of benzothiophene or metabolites, or benzothiophene being a poor substrate, the products formed (2- and 5-carboxybenzothiophene) indicated that the initial enzyme could nonspecifically attack either the benzene or thiophene ring. As for naphthalene, the C_1 unit was derived from bicarbonate, as revealed in ^{13}C radiolabeling experiments (22). In similar experiments with [^{13}C]bicarbonate and 2-[^{14}C]methylnaphthalene, the formation of 2-naphthoic acid via methyl group oxidation was observed in a sulfate-reducing consortium. Also, the presence of 2-methynaphthalenes suggests an alternative metabolic pathway (594).

To date, the mechanism of benzene activation leading to its anaerobic degradation has not been elucidated because no pure cultures have yet been isolated for study. Recently, two *Dechloromonas* strains (RCB and JJ) of the β-proteobacteria that mineralize benzene with nitrate as the electron acceptor have been isolated (123), and elucidating the genetics and biochemistry of this metabolism is an area that deserves attention.

The diversity and unique properties of the anaerobic hydrocarbon-utilizing bacteria are areas that are in need of more work. While difficult, greater focus on isolating and characterizing the enzymes involved in anaerobic hydrocarbon metabolism is required. Futhermore, uptake, efflux, and chemotaxis, areas only recently explored for aerobes, are topics so far untouched in the anaerobic realm. A balanced shift from molecular biology back to enzymology and protein biochemistry is a move that would benefit the understanding of hydrocarbon metabolism in all areas.

BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO HYDROCARBONS

The molecular and biochemical basis of microbial behavior and physiological responses to hydrocarbons and the impact of these responses on bioremediation have been neglected until very recently. Relatively speaking, the metabolic pathways driving the activation of hydrocarbons into central metabolic pathways are well understood, while behaviors and responses are not appreciated beyond a general observational level. However, these phenomena are essential for allowing hydrocarbon-metabolizing organisms to avoid toxic effects, to access poorly soluble substrates, and, in some cases, to bring very large substrates into the cell. This section will examine some of the recent research into the biochemical mechanisms that control responses to hydrocarbons in an effort to suggest that responses such as changes in membrane architecture, active uptake and efflux, and chemotaxis are all of paramount importance and, in some cases, may be coordinately controlled in order to allow metabolism to take place.

Membrane Alterations, Uptake, and Efflux

Given the hydrophobic nature of the area between the monolayers of the cytoplasmic membrane and, in gram-negative bacteria, of the outer membrane, it is not surprising that lipophilic molecules such as hydrocarbons partition there. In 1995, Sikkema et al. (566) published an extensive review on the mechanisms of membrane toxicity of hydrocarbons for a variety of organisms. They outlined the toxicity of lipophilic compounds, including hydrocarbons (alkanes, cyclic hydrocarbons), alcohols, phenols, and other antimicrobials. Briefly, hydrocarbons tend to reside in the hydrophobic area between membrane monolayers in the acyl chains of phospolipids, with partitioning being related to the octanol-water partition coefficient of the lipophilic compound. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (143, 251, 566).

In terms of general stress responses, bacteria may form biofilms, alter their cell surface hydrophobicity to regulate their partitioning with respect to hydrocarbon-water interfaces or, in gram-negative bacteria, gain protection from hydrophilic lipo-

polysaccharide components that offer high transfer resistance to lipophilic compounds. In addition, energy-dependent repair mechanisms may be used to compensate for losses in membrane integrity resulting from the partitioning of lipophilic compounds. For example, membrane fluidity can be decreased through increased membrane ordering by affecting *cis*/*trans* phospolipid isomerizations, by decreasing unsaturated fatty acid content, and by altering phospholipid head groups (297, 501, 566, 617, 659). These changes may be associated with an overall increase in phospholipid content and increased phospholipid biosynthesis in solvent-stressed cells (484).

These alterations serve to produce a physical barrier to the intercalation of hydrocarbons in membranes, thus offsetting the passive influx of hydrocarbons into the cell. It is generally believed that hydrocarbons interact with microorganisms nonspecifically and move passively into the cells (45). Of course, hydrocarbon-degrading microorganisms must necessarily come in contact with their substrates before any transport, either active or passive, may take place. Traditionally, three modes of hydrocarbon uptake are cited to describe how hydrocarbon-metabolizing organisms come in contact with their substrates. However, since uptake implies an active movement of substrate across the cell membrane, a more accurate nomenclature for the initial stages of cell-substrate interaction may be hydrocarbon access (631). While microorganisms may contact water-solubilized hydrocarbons, decreasing solubility with increasing molecular weight is restrictive (91). Two additional, perhaps more widespread modes of hydrocarbon accession are direct adherence to large oil droplets and interaction with pseudosolubilized oil (67). For example, Van Hamme and Ward (631) described a *Rhodococcus* strain that grew directly on crude oil droplets and could be removed with the addition of exogenous chemical surfactant, while a *Pseudomonas* strain required surfactant-solubilized oil to efficiently access hydrocarbons. In *P. aeruginosa*, hydrocarbon solubilization and micellar transport control hexadecane biodegradation during biosurfactant-enhanced growth (552). Similarly, encapsulating solid *n*-C18 and *n*-C36 in liposomes increased growth and biodegradation by a *Pseudomonas* sp., indicating that cell-liposome fusion may deliver encapsulated hydrocarbons to membrane-bound enzymes (427).

Only a limited number of studies conclusively indicate that active hydrocarbon uptake into bacterial cells occurs. Naphthalene uptake by *P. putida* PpG1 appears to be nonspecific, as there is no inhibition by protein inhibitors or iodacetamine and no requirement for specific naphthalene degradation gene expression (45). Similarly, phenanthrene uptake by *Pseudomonas fluorescens* LP6a appears to be passive, in contrast to the observed energy-dependent phenanthrene efflux (84). With respect to active transport, proton motive force uncouplers have been shown to apparently decrease both *n*-hexadecane (46) and naphthalene (660) uptake, which could indicate that energy-dependent uptake is important in some strains. In these two studies, the fact that the strains being studied could metabolize the substrates over the long incubation times complicates the separation of phenomena related to transport, metabolism, and growth. Probably the best observational evidence for energy-dependent alkane uptake is the case of *Rhodococcus erythropolis* S+14He, which preferentially accumulates *n*-hexadecane from hydrocarbon mixtures (327).

Recently, Story et al. (587) identified a gene (*pbhD*) in *Sphingomonas paucimobilis* var. EPA505 that is necessary for fluoranthene metabolism and has homology to the gene pyruvate phosphate dikinase (*ppdK*), a gene involved in glucose uptake in prokaryotes and plants. The authors postulated that *pbhD* may be involved in the uptake of flouranthene catabolites that leak from the cell, although no experiments were performed to verify this. Even though direct molecular evidence for active uptake has not been presented, it would not be surprising to find energy-dependent pumps that transport hydrocarbons into the cell. The presence of hydrocarbon inclusions, of both pure and partially oxidized alkanes, for example (46, 274), indicates that these substrates can be accumulated against a concentration gradient, presumably an energy-dependent process. In addition, as has been observed for 2,4-dichlorophenoxyacetate (244) and 4-hydroxybenzoate (245) metabolism, uptake and chemotaxis may be coordinately controlled at the molecular level.

Mechanisms of tolerance. While an undisputed molecular mechanism for active hydrocarbon uptake is not yet available, excellent descriptions of active hydrocarbon efflux from bacterial cells have been presented in the last 7 years. In their review, Sikkema et al. (566) stated that "there is no precedent why active excretion systems should not play a role in lowering the concentrations in the cytoplasmic membrane (and cytoplasm) of toxic lipophilic molecules." Since that time, two *Pseudomonas putida* strains (DOT-T1E and S12) have been characterized in great detail, both physiologically and genetically, with respect to their ability to thrive in the presence of hydrocarbons. The most notable advance in this area has been the molecular characterization of active solvent efflux pumps for aromatic hydrocarbons (322, 332, 382, 441, 518).

Ramos et al. (501) isolated *P. putida* DOT-T1E, which metabolizes toluene and is capable of growing in the presence of 90% (vol/vol) toluene. In early studies, DOT-T1E was found to increase membrane rigidity by converting *cis*-9,10-methylene hexadecanoic acid to 9-*cis*-hexadecanoic acid and subsequently to the corresponding *trans* isomer. This short-term response typically occurs in less than 1 min upon exposure to toluene. *P. putida* S12, which does not grow on toluene but can tolerate high levels of organic solvents such as styrene (658) and toluene (659), also exhibits *cis*/*trans* isomerizations (659). In the long-term (15 to 20 min) exposure, DOT-T1E decreased the amount of phospatidylethanolamine in the phospolipid polar head groups and increased cardiolipid levels, again increasing membrane rigidity (501). These changes increase lipid ordering to restore membrane integrity and reduce organic solvent partitioning in the membrane. A gene encoding a *cis*/*trans* isomerase, *cti*, which catalyzes the isomerization of esterified fatty acids in phospholipids (mainly *cis*-oleic acid [$C_{16:1,9}$] and *cis*-vaccenic acid [$C_{18:1,11}$]) has been cloned and sequenced in DOT-T1E.

Null mutants exhibited lower survival rates upon toluene shock. In addition, while a longer lag time was observed when mutants were exposed to toluene in the vapor phase, the growth rates for the mutant and the wild-type strain were similar. Thus, the *cis*/*trans* isomerization helped prevent cell damage but was apparently not the most important element in solvent resistance. Cti is constitutively expressed in DOT-T1E and, as expected, is located in the membrane. The *cti* gene is

also found in nonresistant *P. putida* strains and other *Pseudomonas* species (297).

Toluene tolerance in DOT-T1E was found to be inducible by exposure to toluene in the vapor phase, which led the group to postulate that an active solvent exclusion system and metabolic toluene removal afforded some protection (501). Similarly, resistance to antibiotics and solvents such as ethanol was found to increase in S12 with exposure to toluene but not antibiotics (279). In [^{14}C]toluene influx studies, an energy-dependent efflux system was proposed, as less influx was observed in adapted cells, while greater influx was observed in the presence of potassium cyanide, a respiratory chain inhibitor, and *m*-chlorophenylhydrazone, a proton conductor (276). The interruption of toluene metabolism through mutation of the *tod* genes did not affect toluene tolerance in DOT-T1E, suggesting that some other mechanism of tolerance was involved (440). Indeed, active solvent exclusion systems, have been characterized in these two strains.

The *srpABC* (solvent resistance pump) genes of *P. putida* S12 were the first to be cloned and unambiguously shown to be responsible for toluene efflux (322). The pump consists of SrpB (inner membrane transporter), SrpC (outer membrane channel), and SrpA (periplasmic linker protein) and is homologous to the proton-dependent multidrug efflux systems of the resistance/nodulation/cell division (RND) family of pumps, which export antibiotics metals, and oligosaccharides. These pumps have been well reviewed by Paulsen et al. (477).

Induced by aromatic and aliphatic solvents and alcohols, the efflux system encoded by *srpABC* is proton dependent and does not pump antibiotics or other substrates of multidrup resistance pumps (277). Unlike cis/trans isomerisations, which can be a general stress response (251), the *srpABC* genes are not induced by extremes of pH, temperature, salt, organic acids, or heavy metals (323). These adaptation mechanisms are energy consuming and have been shown to decrease growth rates and yields while increasing maintenance energy and lag times (278). Presumably, the increased energy consumption may also result from solvent-mediated membrane uncoupling and disruption of energy-transducing proteins.

The first efflux pump in DOT-T1E was found by producing a toluene-sensitive, octanol-tolerant mutant (DOT-T1E-18) by Tn*5-phoA* mutagenesis with a gene knockout homologous to the drug exclusion gene *mexB*, which is a member of the efflux pump family of the resistant modulator type (502). The gene was named *ttgB* for toluene tolerance gene. Solvent exclusion testing with 1,2,4-[^{14}C]trichlorobenzene showed that increasing toluene concentrations increased the amount of radiolabel in the membranes. In addition, the pump was shown to be specific, as DOT-T1E is sensitive to benzene but not *m*-xylene. Given the fact that the mutant exhibited low levels of survival when toluene was delivered in the vapor phase, it was postulated that at least two efflux pumps were present, one constitutive and one inducible.

Indeed, three toluene efflux pumps have ultimately been found in DOT-T1E (441, 518). This is not without precedent, as *P. aeruginosa* has at least three RND antibiotic efflux pumps, which also accommodate organic solvents: MexAB-OprM, MexCD-OprJ, and MexEF-OprN (381, 382). The first pump in DOT-T1E, *ttgABC*, is a constitutive efflux pump controlled by *ttgR*, which produces a transcriptional repressor for the *ttgABC* operon, which in turn is controlled by another repressor belonging to the Lrp family of global regulators. In this case, TtgR is expressed at high levels in the presence of toluene, which in turn reduces TtgABC expression (158). The second pump, *ttgDEF*, is found adjacent to the *tod* genes and is expressed in response to toluene and styrene. Unlike *ttgABC*, *ttgDEF* does not appear to efflux antibiotics and is closely related but not identical to the toluene efflux pump *srpABC* of *P. putida* S12.

The third pump, *ttgGHI*, is expressed constitutively at high levels from a single promoter and, if grown with toluene, is expressed at higher levels from two promoters: one a constitutive promoter and a second, overlapping, inducible promoter (518). *ttgG* encodes the periplasmic lipoprotein that is anchored to the inner membrane and, along with the inner membrane pump encoded by *ttgG*, forms the putitive translocase. *ttgI* encodes the outer membrane protein that may form a channel into the periplasmic space (518). In order to make DOT-T1E sensitive to toluene shock and to eliminate its ability to grow with toluene in the gas phase, mutations had to be introduced in all three pumps. Mutation studies showed that TtgABC and TtgGHI pump toluene, styrene, *m*-xylene, ethylbenzene, and propylbenzene. TtgDEF only removes tolene and styrene.

Overall, it appears that efflux pumps in *Pseudomonas* spp. can be divided into three general groups: those that pump organic solvents, those that pump antibiotics, and those that pump both. Kieboom et al. (321) recently described an active antibiotic efflux pump in S12 (ArpABC) which does not pump solvents. This is in contrast to the MepABC pump in *P. putida* KT2442 (206) and the Mex pumps in *P. aeruginosa* (382), which pump both solvents and antibiotics. Furthermore, much will be gained if efflux pumps for other hydrocarbons and for other microorganisms are studied in detail and compared to known systems. Further research at the protein level will be required for many systems, as comparative studies will help to unravel the factors affecting pump specificity, to understand what forces govern substrate recognition, and to see if and how pump receptors are able to regulate other behaviors such as taxis, the final behavior to be discussed here.

Taxis

Motile bacteria are able to control their spatial position with respect to various stimuli such as chemicals, light, and redox potential by a variety of mechanisms. Chemotaxis is the response to a stimulus independent of cellular metabolism through chemoreceptors. On the other end of the spectrum, a microorganism may exhibit metabolism-dependent energy taxis, where behavioral responses are to changes in energy levels in the cell and not the stimulus itself. Finally, there are cases when the chemotactic behavior is in response to substrate transport across the membrane (604). The molecular and biochemical nature of these behaviors has been relatively well studied for water-soluble substrates and has only recently been explored in hydrocarbon-degrading bacteria.

One can imagine that movement away from a hydrocarbon plume could reduce toxic effects or that movement towards a

water-insoluble substrate such as naphthalene could be advantageous in poorly mixed field situations. Indeed, Marx and Aitken (410) used a capillary assay (409) to show that *Pseudomonas putida* G7 catalyzed naphthalene degradation at faster rates in unmixed, heterogeneous systems than did mutants deficient in either motility or naphthalene chemotaxis. In mixed systems, the naphthalene degradation rate was identical for the wild-type and mutant strains.

P. putida G7 possesses the NAH7 catabolic plasmid for the *meta*-cleavage of aromatic hydrocarbons (226, 227). The plasmid includes the *nahY* gene, encoding a 538-amino-acid membrane protein whose C terminus resembles that of chemotaxis transducer proteins (i.e., methyl-accepting chemotaxis proteins). This indicates that NahY may be a chemoreceptor for naphthalene or naphthalene metabolites (227), but neither the molecular nature of binding nor the cascade of responses that occur following binding has been studied.

Pseudomonas putida RKJ1 possesses an 83-kb plasmid for naphthalene metabolism through salicylate (538). A Nap^- Sal^+ mutant was chemotactic towards only salicylate, while a Nap^- Sal^- mutant exhibited no chemotaxis. This suggests the presence of a metabolism-dependent energy taxis in this strain. Thus, a change in the redox potential or cellular energy level in the cell probably provides the signal for chemotaxis. Alternatively, a membrane-bound or intracellular chemoreceptor may recognize naphthalene or salicylate degradation products.

To date, no reports describing the molecular basis for alkane chemotaxis have appeared. However, van Beilen et al. (627) detected *alkN* in the 9.7-kb region between *alkBFGHJKL* and *alkST* in *P. putida* GPo1, which encodes a protein with 30% sequence similarity to methyl-accepting transducers such as the one found in strain G7 (227). As GPo1 is not very motile, the functionality of the gene is difficult to study.

Overall, taxis in relation to petroleum hydrocarbons has been neglected, and the area is ripe for study. First of all, more examples of tactic behavior to hydrocarbons are required in other genera and with different hydrocarbons in order to appreciate the diversity of responses. Second, when putative chemoreceptors are detected by gene sequencing, systematic studies of purified proteins are required in order to understand the key molecular interactions that take place to allow a cell to detect a particular chemical. Third, the mechanisms by which chemoreceptors translate signals induced by hydrocarbons into cellular responses and their impact on overall cellular biochemistry would allow the integration of this behavior, and all of the behaviors discussed here, into a larger picture of hydrocabon-metabolizing organisms. Recent developments for the large-scale and nearly real-time monitoring of gene expression in live cells with green fluorescent protein promoter fusions (300, 579) will allow this type of integrating study. Finally, understanding the true role of chemotaxis during remediation needs more attention if we are going to understand the impact of taxis on biofilm formation, substrate access, and avoidance of toxic substances. Recent developments in tracking live bacterial cells with advanced imaging technologies (559) could be combined with gene expression technologies and traditional measurements of hydrocarbon degradation (258) to study these questions.

MICROBIAL COMMUNITY DYNAMICS

Ecologically, hydrocarbon-metabolizing microorganisms are widely distributed. Difficulties arising during attempts to characterize natural microbial communities impacted by petroleum hydrocarbons are exacerbated by the myriad of individual substrate and metabolite interactions possible. Despite the intricacies, tools are being developed in an attempt to better appreciate microbial abundance and distribution in natural environments in the hopes of associating community structures with ecosystem functions. The rationale for undertaking such analyses includes describing the role of microorganisms in the genesis of petroleum over geological time (398, 465), evaluating the long-term effects of petroleum pollution (386), developing and evaluating waste remediation approaches (298, 565), tracking the enrichment of pathogenic microorganisms during remediation (56, 197), and controlling deleterious microbial activities during petroleum production (165, 166).

Approaches to cataloguing microbial diversity and community function can be broadly divided into culture-dependent and culture-independent methods, both of which may include genetic characterization techniques. Traditional culture-dependent methods are the most familiar and are based on differential morphological, metabolic, and physiologic traits. These include isolation and cultivation on solid media, most-probable-number (MPN)-style liquid assays, and more recently, Biolog substrate utilization plates. Culture-independent methods for community analysis began with direct examination of metabolically active microorganisms with differential stains such as 4′,6′-diamidino-2-phenylindole, (INT)-formizan and CTC, fluorescence in situ hybridization, and bulk analysis of total protein banding and phospholipid fatty acid analysis.

With rapid expansions in the field of molecular genetics, a host of PCR-based approaches have emerged to study specific microorganisms or groups of microorganisms and specific genes and to evaluate overall community profiles. Methods to evaluate community profiles include denaturing and temperature gradient gel electrophoresis, ribosomal intergenic spacer analysis, single-strand conformation polymorphism, internal transcribed spacer-restriction fragment length polymorphism, random amplified polymorphic DNA, and amplified ribosomal DNA restriction analysis (317). Recently, developments in the use of DNA microarrays have attracted the attention of environmental microbiologists for more rapid throughput to allow the tracking of thousands of genes at one time (146).

A few examples of community studies involving petroleum applications are discussed here in order to highlight the utilities and limitations of the various methods (Table 3).

Culture-Based Methods

Traditional culture techniques have yielded valuable information about microbial interactions with hydrocarbons in the environment. However, one must keep in mind that only a small fraction of microorganisms can currently be cultured from environmental samples, and even if a microorganism is cultured, its role in a community and contribution to ecosystem function are not necessarily revealed. This was especially evident in early studies, where catalogues of microorganisms were compiled based on conventional isolation and plating tech-

TABLE 3. Utility and limitations of some community analysis methods

Type	Example	Utility	Limitations
Culture dependent	Plating	Isolates obtained for further study	Only a small proportion of community detected, isolates not necessarily reflective of a specific metabolic function
	MPN	Metabolic function of interest detected	No isolates obtained for further study, selective media may limit proportion of community detected
	Biolog	Overall metabolic activity detected, rapid and easy to use	No isolates obtained for further study, selective media may limit proportion of community detected, may not include substrates of interest, sensitive to inoculum size and incubation effects
Culture independent	Phospholipid fatty acid analysis	Changes in fingerprint can indicate change in community structure	No isolates obtained for further study
	Protein banding	No selection pressure if extracted directly	No measurement of community function, difficult to link fingerprints to specific microbial groups
	Fluorescence in situ hybridization	Spatially visualize specific microorganisms in an environment, no bias from culture media	Not necessarily detecting active microorganisms, laborious technique
	Staining for active microbes	Enumerate live microorganisms, no bias from culture media	Does not differentiate microorganisms with catabolic activity of interest
	RSGP[a]	Quantitative analysis of specific microorganisms in environmental samples, no bias from culture media	Limited to those microorganisms included in the screen
	PCR followed by gel electrophoresis	No bias from culture media, can identify microorganisms by sequencing resolved bands, bulk changes in community structure detected	Differential DNA or RNA extraction from different cells, differential amplification during PCR, no information on activity; no isolates for study
	Probes for specific metabolic genes	Detect genes with function of interest, mRNA detection can reveal information about expression	Limited to known genes, activity cannot be inferred from presence of genes alone
	Promoter-reporter systems	Gene expression detected, treatment effects on total cell function can be monitored	Nature of promoter must be known, easier to apply when whole genome sequences are available, monitors only those strains with reporter genes inserted

[a] RSGP, reverse sample genome probing.

niques. These studies documented a broadly distributed and diverse collection of bacteria, yeasts, and fungi capable of hydrocarbon utilization (29), and similar contemporary investigations continue to catalogue microbial communities from hydrocarbon-impacted environments around the world (28, 106, 272, 411, 506, 577).

If one is interested either in reporting an isolated microorganism as having hydrocarbon-metabolizing abilities or in performing enumerations of hydrocarbon-degrading microorganisms, it is essential to include proper controls. Ample evidence is available to illustrate that non-hydrocarbon-degrading microorganisms will develop on agar plates prepared with solid, liquid, or volatile hydrocarbons due to the presence of utilizable carbon even in purified agarose (60, 504). In an evaluation of mineral agar plates with and without toluene-xylene fumes, it was revealed that little selection was provided against non-toluene- and non-xylene-degrading bacteria. Despite the caution to incubate plates with and without hydrocarbon, studies with oil agar to enumerate hydrocarbon-degrading bacteria without reporting proper controls can still be found. This type of report should be examined with care.

In an attempt to overcome the problem with trace carbon in agar preparations, some researchers turned to the use of silica gel as a solidifying agent. However, this tedious procedure has not enjoyed widespread use. If isolates are not required, a rapid MPN test (sheen-screen) with tissue culture plates can be employed for nonvolatile hydrocarbons based on the formation of emulsions, avoiding the problem of trace carbon contamination altogether (77). A similar assay to screen for hydrocarbon degraders based on a redox indicator has been described (236) and combined with the sheen-screen to produce an MPN assay based on both emulsification and respiration (633).

Numerous studies have attempted to describe microbe-microbe and microbe-hydrocarbon interactions by extrapolating from detailed laboratory studies with isolates from hydrocarbon-contaminated environments. For example, evaluations of functional and physiological isolate groupings have been carried out in an effort to quantify the oil emulsification abilities and type of hydrocabon accession mode used by environmental isolates (67). Researchers have also constructed simplified consortia containing several well-defined strains in an effort to

identify specific processes that may be important in environmental settings.

In a recent study evaluating 10 strains enriched with phenanthrene as the sole carbon and energy source (7), isolates were examined without confounding interactions associated with complex media, substrates, and microbial mixtures. Strains from eight sites were able to metabolize PAHs with two to five rings following growth on phenanthrene. In terms of metabolism (oxidation, mineralization, or removal), each strain was unique with respect to substrate specificity, and all could oxidize at least one intermediate of the two known PAH degradation pathways (salicylate or phthalate). Despite widespread ability to metabolize benz[*a*]anthracene, chrysene, and benz[*a*]pyrene, none of the strains could mineralize pyrene alone. This led the authors to conclude that unique cometabolic processes are required for pyrene removal in natural environments. This is a common conclusion that, while probably correct, is typically unsubstantiated by any direct evidence or description of the specific processes involved.

Komukai-Nakamura et al. (340) evaluated various mixtures of an alkane-utilizing *Acinetobacter* spp. and a *Rhodococcus* sp., an alkylbenzene-degrading *Pseudomonas putida*, and a phenanthrene-utilizing *Sphingomonas* sp. in an attempt to elucidate how alkane- and aromatic-degrading microorganisms interact. The degradation of Arabian light crude oil was monitored, and a combination of the *Acinetobacer* sp. and *P. putida* was as effective as a mixture of the four microorganisms, degrading 40% of the saturates and 21% of the aromatics. Respirometry showed that *P. putida* was able to evolve CO_2 from unidentified metabolites of *n*-octylbenzene produced by the *Acinetobacter* sp. Many bioremediation companies offer such mixed cultures for sale to cope with environmental pollution (342), but third-party testing of such products has not proven them to be more effective than autochthonous microbial communities once additional nutrients and sorbents are removed (611, 638). Standard assay procedures with simple consortia are being developed for Environment Canada (199, 198) and the U.S. Evironmental Protection Agency (232) in order to test such products.

These types of study are essential for understanding general mechanisms but do not reveal environmental importance. To achieve a greater understanding, the molecular biology and biochemisty of the processes need to be understood in detail so that gene expression can be correlated to activity. For example, using green fluorescent protein fusions, Holden et al. (258) showed that, in contrast to liquid cultures, expression of genes for rhamnolipid and PA bioemulsifying protein did not improve biodegradation of *n*-hexadecane in an unmixed sand culture. Instead, adherence to the hydrocarbon-water interface was more important for biodegradation.

Aside from isolating and identifying microorganisms present in hydrocarbon-impacted environments, descriptions of microbial communities have been based solely on functional characteristics. Normally based on MPN assays, dividing communities into physiological types is best served if numerous selective media are used and associated with relevant site characteristics. The MPN has appeared to be particularly useful for studying anaerobic systems, as it is sensitive, even when slow-growing anaerobes are being studied. Kämpfer et al. (301) monitored in situ bioremediation of a waste oil-contaminated site subjected to various bioremediation treatments. Both groundwater and soil samples were taken with the aim of correlating microbiological and chemical data to assess bioremediation potential. Microorganisms were divided into the following classes: methylotrophic, facultative anaerobes, denitrifiers, sulfate reducers, oil-degrading denitrifiers, and anaerobic vacuum gas-oil degraders. In addition, 3,466 bacterial isolates (42.5% gram-positive) from R2A agar were identified, with >70% being previously reported as hydrocarbon degraders. While this is an impressive number of isolates, there is no indication of how important these isolates are in that particular environment. A separate study of a crude oil-contaminated aquifer (51) used a similar MPN approach to study ecological succession, microbial nutrient demands, and the importance of free-living versus attached populations. MPN determinations of aerobes, denitrifiers, iron reducers, heterotrophic fermenters, sulfate reducers, and methanogens were used. The dominant physiological types were consistent with the known geochemical evolution of the contaminant plume, from iron-reducing to methanogenic.

In Antarctica, Delille et al. (145) examined seasonal changes in the functional diversity of ice bacteria over 9 months in uncontaminated, contaminated, and treated (Inipol EAP22 fertilizer) plots. Total bacteria (acridine orange) saphrophytes, and hydrocarbon-utilizing bacteria (MPN) were assayed. In all cases, changes in total bacterial abundance, reaching a minimum in the winter ($<10^5$ cells ml^{-1}), were correlated with seasonal variations. Following crude oil or diesel fuel contamination, bacterial counts increased, with increases in oil-degrading bacteria from 0.001% to 10%. Both saprophytic and oil-degrading bacteria increased with Inipol addition. In contrast, the underlying seawater showed limited variation between control and contaminated plots. In lieu of MPN assays, direct immunofluorescence and enzyme-linked immunosorbent assay have been used for nearly real-time quantification of hydrocarbon-degrading organisms (76). Immunodetection was shown to be applicable to complex sample matrices for rapid field evaluation. Antibody mixtures of sufficient specificity could potentially be developed to target specific microbial groups, although, in most situations, tracking the expression of specific genes involved in hydrocarbon metabolism would be of greater utility.

The most effective uses of an MPN approach, or indeed any approach to characterize a petroleum-impacted microbial community, has been realized when evaluating the role of a particular microbial group during remediation. For example, during enhanced oil recovery by water flooding, wells are often contaminated with hydrogen sulfide-producing sulfate-reducing bacteria that result in the souring of sweet crude oils. Biocides have often been found to be ineffective in controlling this problem, while nitrate addition has been used with some success (165, 166, 607). Eckford and Fedorak (165, 166) undertook an MPN-based study of some western Canadian oil field waters to show that nitrate addition stimulates the growth of heterotrophic nitrate-reducing bacteria that outcompete sulfate-reducing bacteria, presumably due to more favorable metabolic energetics. Nitrate-reducing bacteria have been neglected in the study of petroleum reservoirs (398), which illustrates that a circular approach to community studies, whereby

non-culture-based approaches lead to the development of new isolation techniques and vice versa, is recommended.

Total community analyses have been carried out with phospholipid fatty acid analysis profiles and Biolog substrate utilization patterns. In Australia, phospholipid fatty acid analysis profiles were evaluated as a method to provide insight into the monitoring-only approach during management of a gasoline-contaminated aquifer (202). Principal-component analysis did not reveal any clear groupings with respect to an aromatic hydrocarbon plume, and phospholipid fatty acid profiles were rejected as expensive and technically difficult for their purpose. A similar study (183) used total phospholipid fatty acid profiles to evaluate microbial community structure and biomass levels in a JP-4 jet fuel-contaminated aquifer. Aerobic and anaerobic zones were examined, and specific fatty acids were used in an attempt to draw conclusions with respect to the presence of aerobes and anaerobes, but overall, phospholipid fatty acid patterns are not sufficiently powerful to provide solid data about the presence of specific microorganisms in a community, let alone provide insight into their function.

Protein banding pattern analysis as a method to infer the function of isolates from a contaminated aquifer was found to suffer from the same limitations when evaluated by Ridgway et al. (513). A total of 297 isolates were screened for the ability to use gasoline vapor as a sole carbon and energy source and were pooled into 111 groups based on the usage pattern of 15 different volatile organic hydrocarbons. Following identification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns were used to regroup the isolates. Fifty-one groups were resolved that partitioned into two broad classes (metabolically diverse and metabolically restricted), but catabolic activity could not be predicted.

Berthe-Corti and Bruns (57) used Biolog substrate utilization patterns to evaluate the functional diversity of microbial communities in continuous-flowthrough cultures treating C_{16}-contaminated intertidal sediments. Standard dissolved oxygen and dilution rate effects typically used in in situ remediations were implemented because it is desirable to determine if adaptations to low oxygen are due to changes in microbial community structure or metabolic adaptations of specific populations. Measurements of C_{16} degradation, product formation, oxygen consumption, total heterotrophs, and MPN determinations of nitrate reducers, sulfate reducers, and C_{16}-utilizing bacteria were combined with Biolog data. It was observed that substrate utilization became more limited, especially at low dissolved oxygen (0.4%) levels. Other parameters (C_{16} degradation, protein production, and oxygen consumption) increased with dilution independently of dissolved oxygen. Overall, the level of dissolved oxygen (80% or 0.4%) appeared to dictate the structure of the microbial community.

Lindstrom et al. (386) evaluated the long-term effects of a 1976 experimental sub-Arctic oil spill in Alaska by examining soil population structure and community-level metabolism. No differences in total bacterial numbers or soil carbon mineralization were detected, while hydocarbon degraders (based on the sheen-screen assay) were elevated at the oil-contaminated site. Nitrogen mineralization and metabolically active microorganisms were abundant at the contaminated site. A kinetic analysis of the Biolog results was used to avoid problems with inoculum density and time-of-reading effects. Taken together, the evidence was interpreted to conclude that the oil resulted in diminished microbial population diversity and selection for metabolic generalists even after extended exposure times. However, the importance of the observations in terms of overall ecosystem function is difficult to determine.

Culture-Independent Approaches

At this time, we are beginning to understand the astonishing diversity of microbial populations and communities in the environment. Coming to grips with the inherent variability in microbial communities over space and time, even in the absence of petroleum hydrocarbons, remains a major challenge. Culture-independent approaches to microbial community analyses have recently enjoyed a surge in popularity as new techniques have been developed and are available in most major research institutions. Molecular descriptions of microbial communities now dominate the literature in all areas of microbial ecology, not just petroleum microbiology.

To be successful in the future, rapid automated systems will be required to process and evaluate vast quantities of data in order to subtract background variability. Even then, care must be taken to realize that, while molecular methods are powerful and attractive, the genetic composition of a community cannot be used to extrapolate ecosystem function. Kent and Triplett (317) summarized the current state of microbial community analysis succinctly: "The current era of investigation can be viewed as the descriptive phase, which is necessary prior to a testing phase where we will learn the role and perhaps the functional redundancy of the perhaps hundreds of millions of operational taxonomic units in soils on earth."

A few of the recent studies will be discussed here, and it is important to note that most studies involving culture-independent characterization of petroleum-impacted microbial communities have included other measures of microbial activity with culture-dependent methods. This is a requirement for making sense of data generated from culture-independent methods and to allow the development and evaluation of new methods.

Bulk measurements of total community DNA in a manner analogous to phospholipid fatty acid analysis and protein banding patterns have been used in an attempt to detect perturbations and changes in petroleum-impacted environments. Unlike phospholipid fatty acid analysis, specific microorganisms can be identified if the genetic material is extracted from each individual band following elecrophoresis and then sequenced. This practice is time-consuming, and identification results, while intriguing, are often left without further attempts to isolate the observed organisms.

Shi et al. (560) used Domain probe analysis to examine community structure in pristine and fuel-contaminated aquifers. The predominantly bacterial populations were further divided (43 to 65% β- and γ-proteobacteria, 31 to 35% α-proteobacteria, 15 to 18% sulfate-reducing bacteria, 5 to 10% high G+C). Physical-chemical data and the lack of members of the *Archaea* suggest that methanogenesis was not occurring in the aquifer. Øvreås et al. (468) used denaturing gradient gel electrophoresis (DGGE), sequencing, and DNA reassociation plots in combination with measurement of methane and methanol oxidation measurements to show a decrease in diversity

with a concomitant increase in known methanotrophs upon methane perturbation of agricultural soils.

MacNaughton et al. (394) used 16S rRNA PCR-DGGE and phospholipid fatty acid analysis to identify populations responsible for decontamination while evaluating oil spill bioremediation techniques and to help define an endpoint for substrate removal. Phospholipid fatty acid analysis, PCR-DGGE patterns, degradation rates, and hydrocarbon degraders (MPN) were similar for plots with nutrient and with nutrient plus inoculum. Complex banding patterns and low reproducibility were encountered, along with some disagreements between phospholipid fatty acid analysis and DGGE analysis. However, two novel bands, closely related to *Flexibacter-Cytophaga-Bacteroides* were detected in all nutrient-amended sites. Their contribution to enhanced degradation remains speculative. Roony-Varga et al. (521) also used a mixed approach to evaluate anaerobic benzene degradation in a petroleum-contaminated aquifer. Phospholipid fatty acid analysis, MPN-PCR, and DGGE of 16S ribosomal DNA along with selective enrichment and biodegradation studies were used. Increased diversity at contaminated sites was observed along with higher phospholipid fatty acid contents. MPN-PCR indicated that *Geobacteriaceae* spp. were important at the site, which disagreed with phospholipid fatty acid profiles. This may be an indication that, while phospholipid fatty acid analysis can be useful for identifying isolated microorganisms, its utility as a tool for extrapolating the identity of individual community members from a total phospholipid fatty acid pattern is limited.

To date, community characterizations have been, for the most part, applied to field situations. Hydrocarbon-contaminated or impacted sites rather than fermentor-based treatment systems have been the target of characterization. Thus, this type of system may be useful for developing methods in a more controlled environment. Colores et al. (128) studied surfactant effects on C_{16} and phenanthrene degradation by a mixed culture in laboratory microcosms by respirometry, 16S rRNA DGGE, and culture techniques. They found that surfactant levels close to the critical micellization in soil inhibited mineralization and shifted the community from *Rhodococcus* and *Nocardia* populations to *Pseudomonas* and *Alcaligenes* species able to degrade both surfactant and hydrocarbon. Of 60 isolates, 11 unique DGGE banding patterns were obseved, three of which (*Rhodoccocus*, *Psuedomonas*, and *Alcaligenes*) corresponded to major bands from the whole-community analysis.

It is apparent that total community approaches such as 16S rRNA DGGE banding patterns are not the end-all in understanding microbial communities or providing sufficient power to address specific hypotheses (565). More information is often available when gene probes for specific isolates, genotypes, or metabolic activities are used, and approaches to achieve this are being applied in both aerobic and anaerobic systems (117, 128, 156, 257, 304, 478, 514, 582, 609).

An excellent example of this has come out of Voordouw's laboratory at the University of Calgary. That group has published extensively on the use of molecular methods for the quantitative analysis of sulfate-reducing bacterial communities in oil fields (263, 605, 646). Sulfate-reducing bacteria play a key role in anaerobic corrosion in oil and gas fields, and elucidating their modes of action is important to oil companies. To this end, metabolic activity tests are useful but do not provide information about specific species. Early work showed that gene probes based on the [Fe], [NiFe], and [NiFeS] hydrogenases could be used to identify *Desulfovibrio* spp. (648).

The observation that many specific hydrogenase probes failed to hybridize with sulfate-reducing bacterial isolates led to the development of reverse-sample genome probing (645). This technique allows the total DNA from a community to be quantitatively (649) analyzed in a single step. The proportion of the community being analyzed is related to the quantity of probe in the master filter, and a quantitative approach has been developed (649), and adding probes for non-sulfate-reducing bacteria to a filter is straightforward (647). Biofilm formation (649), nitrate injection (607), and diamine biocide (606) effects with respect to community composition and functional properties have been described. The approach has also been used for evaluating hydrocarbon-degrading bacteria in soil exposed to dicyclopentadiene (556), although it must be kept in mind that important groups of organisms may be missed with this method and that the presence of a specific microorganism does not indicate that it is active.

From a remedial perspective, tracking specific genes expected to be present in isolates from hydrocarbon-impacted environments may be more useful at this time, especially if workable methods for mRNA can be developed. Early work with gene probes following the *Exxon Valdez* spill revealed that bacterial populations containing both the *xylE* and *alkB* genes could be deteced in environemental samples (578). In laboratory columns, proportions of *xylE* and *ndoB* (polycyclic aromatic hydrocarbon degradation) populations from an aquifer community were monitored during degradation of creosote-related PAHs (261). Isolates grown on tryptone-yeast extract medium were probed, and it was found that *p*-cresol addition resulted in a 100-fold increase in total culturable bacteria, with a threefold increase in *xylE*- and *ndoB*-positive populations. Langworthy et al. (359) found *nahA* and *alkB* in higher frequencies at PAH-contaminated sites, although these genes, along with *nahH* and *todC1*/*C2* were detected at pristine sites as well. Laurie and Lloyd-Jones (365) recently used competitive PCR to illustrate that the newly descibed *phn* genes of *Burkolderia* sp. strain RP007 may have greater ecological significance than *nah*-like genes for PAH degradation. The *phn* genes, while encoding the identical biodegradation pathway, have low sequence homology to *nah*, a different gene order, and are present in the organisms that are rarely cultured in the laboratory.

If the biochemistry and genetic diversity are known, gene probe suites have greater potential for accurately evaluating bacterial degradative potential (234, 424), although the application of a small number of probes may be effective if meaningful hypotheses are tested (565). Recent advances in characterizing alkane metabolism in a number of organisms have allowed the production of a variety of primers to detect, for example, the *alkB* gene from *P. putida* GPo1 (573). As more strains are tested and more probes are produced, it is becoming clear that, while different alkane hydroxylases can be found in phylogenetically distant microorganisms (19), many probes will only provide information on the presence of a similar gene in closely related strains. Thus, the usefulness of such gene probes will grow as the diversity of genes responsible for hy-

TABLE 4. Evaluation of various petroleum sludge treatment technologies

Remediation	Technology	Comments
Bioremediation	Bioreactor	Application of natural and specialized microorganisms in controlled environmental and nutritional conditions, high biodegradation rates, accommodates variety of sludges, nonhazardous residues, on-site operation, cost-effective
	Landfarming	Uses natural microbial population and supplements of mineral nutrients, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water, cost-effective
	Biopiling	Uses natural microbial population and supplemented nutrients and air, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water
	Bioventing	A combination of advective soil venting and biodegradation method for in situ treatment of soils, most of the lighter hydrocarbons are volatilized
	Biostimulation/bioaugmentation	Application of mineral nutrients/surfactants and/or microorganisms to stimulate or supplement natural microbial population at contaminated site
	Phytoremediation	Uses plants and rhizospheric microorganisms for the treatment of contaminated soil, potential for removal of petroleum contaminants being evaluated, presumably cost-effective
Physicochemical	Incineration	High-temperature treatment, air pollution risks, expensive control equipment, high capital cost
	Thermal desorbtion	High-temperature oil removal and recovery method from oily solids, high capital and material preparation costs, nonhazardous residues
	Coker	Complicated sludge preparation for coker feed, some oil recovery, high capital and transportation costs
	Cement kiln	Complicated sludge preparation for use of fuel, high material preparation, transportation, and disposal costs
	Solvent extraction	Uses solvents and centrifugation or filtration for the separation of oil from sludges, safety hazard with solvent use, high capital cost

drocarbon metabolism is better appreciated (120, 573, 644, 661, 662, 663).

This field will be greatly advanced if genome projects are initiated to sequence environmentally important microorganisms, including fungi, if the diversity of hydrocarbon metabolic pathways is better characterized, and if tools to monitor gene expression on a large scale are developed (146). Finally, the most important point to recall when embarking on a community-based study is that a clear, testable hypothesis be framed at the outset.

MICROBIAL TREATMENT OF PETROLEUM WASTE

Earlier reviews on hydrocarbon biodegradation have described bioremediìation efforts, including the use of chemicals (surfactants and dispersants) (126, 194, 370, 489, 522, 599). The general importance of relying on the indigenous microbial population, which presumably resists tidal washing by association with oily surfaces rather than on inocula, has been emphasized.

Environmental impacts from the petroleum industry derive from recovery, transport, refining, and product usage. Only 10% of the last is attributed to high-profile marine oil spill catastrophes resulting in shoreline contamination (36, 489). In various operations of production, processing, and storage, large volumes of waste are generated as oily sludges (404). Hydrocarbons bind strongly to solid surfaces, including soils, and remediation of these materials represents a significant challenge. The lighter and often toxic hydrocarbon components tend to volatilize into the atmosphere, reducing air quality and threatening human and animal health. High levels of sulfur compounds are also emitted in petrochemical waste streams, which require treatment. The followling sections will focus on treatment of petroleum-contaminated solids, biofiltration of volatile compounds from air streams, and removal of sulfur compounds from waste streams. Hence, in contrast to earlier reviews which focused on clean-up of contaminated sites, the main emphasis here is on bioprocessing of waste streams.

Treatment of Contaminated Soils and Sludges

Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution because the majority of molecules in the crude oil and refined products are biodegradable and oil-degrading microorganisms are ubiquitous (Table 4) (6, 107, 185). However, abiotic losses due to evaporation, dispersion, and photooxidation also play a major role in decontamination of oil spill environments (211, 535). In the case of in situ subsurface bioremediation processes, the greatest challenges relate to engineering of the subsurface environment so that microbes can thrive there and effectively degrade the contaminants present. Biological methods for processing of oily sludges and oil-contaminated soils in landfarming, biopiling/composting, bioventing, and bioreactor configurations have been well documented (30, 342, 489, 652).

Factors affecting bioremediation. The rate of microbial degradation of crude oil or oil waste depends on a variety of

factors, including the physical conditions and the nature, concentration, and ratios of various structural classes of hydrocarbons present, the bioavailability of the substrate, and the properties of the biological system involved (337, 593, 637, 669, 684). A generalized sequence of petroleum components in order of decreasing biodegradability is represented as follows (268): *n*-alkanes > branched-chain alkanes > branched alkenes > low-molecular-weight *n*-alkyl aromatics > monoaromatics > cyclic alkanes > polynuclear aromatics >> asphaltenes. Predictive models for estimating the extent of petroleum hydrocarbon biodegradation (268) and diffusion-controlled bioavailability of crude oil components (621) have been developed. Properly chosen chemical surfactants may enhance biodegradation (79, 80, 453, 529, 634). The efficiency of processes for degradation of hydrocarbons will also depend on the nature of the hydrocarbon-contaminated material, the environmental conditions, and the characteristics of the microbial population that is present.

Assuming that microbes are present, nutrient availability, especially of nitrogen and phosphorus, appears to be the most common limiting factor (494, 526). Laboratory and field experiments with inorganic nitrogen and phosphate fertilizers and organic fertilizers, including fish bones, fish or animal meal, biosurfactants, and bulking agents, have shown success (68, 241, 371, 372, 428, 446, 458, 512, 635, 640).

Strategies for microbial degradation of petroleum contaminants or wastes manifest themselves in processes having different degrees of complexity and technological requirements. Bioremediation of contaminants in soil by natural attenuation requires no human intervention, whereas implementation of accelerated and controlled bioreactor-based processes may be directed to exploiting microbial technology and bioprocess engineering to optimize the rates and extents of contaminant degradation.

In simple bioremediation systems, which require little or no microbiological expertise, process-limiting factors often relate to nutrient or oxygen availability or the lack of relatively homogeneous conditions throughout the contaminated medium. Microbial growth and degradation processes operating under such conditions are typically variable and suboptimal, leading at best to prolonged degradation cycles (443). Processes are often unreliable, and required contaminant degradation endpoints are often not achieved throughout the medium. These processes tend to ignore the realities of enzyme and cell substrate saturation kinetics, where rates of degradation slow as contaminant concentrations fall, with resulting reductions in the viable microbial population. When contaminants are degraded by cometabolism, early elimination of the cosubstrates, necessary for degradation of these contaminants, can halt the degradation processes. The nonhomogeneous and unpredictable nature of these processes makes them intensive in terms of sampling and analytical activities, as patterns of contaminant removal have to be monitored throughout a three-dimensional grid.

The need for intensive monitoring represents a major justification for the implementation of more optimized biodegradation processes, which ensure contaminants are efficiently biodegraded to defined criteria. Short-term real estate development plans or measures to afford greater protection to the environment or to comply with increasingly stringent environmental regulations require accelerated remediation of contaminated sites. Increasing levels of microbial expertise may be exploited in processes for accelerated transformation of petroleum contaminants and wastes.

TABLE 5. Major biosurfactants produced by microorganisms[a]

Class	Biosurfactant	Microorganisms
Low molecular weight	Rhamnolipids	*Pseudomonas aeruginosa*
	Trehalose lipids	*Arthrobacter paraffineus*
		Rhodococcus erythropolis
		Mycobacterium spp.
	Sophorose lipids	*Candida lipolytica*
		Torulopsis bombicola
	Viscosin	*Psudomonas fluorescens*
	Surfactin	*Bacillus subtilis*
	Polymixins	*Bacillus polymyxa*
	Gramicidin S	*Bacillus brevis*
	Phospholipids	*Acinetobacter* spp.
		Thiobacillus thiooxidans
	Lipopeptides	*Bacillis pumilis*
		Bacillus licheniformis
		Pseudomonas fluorescens
	Polyol lipids	*Rhodotorula glutinis*
		Rhodotorula graminis
	Serrawettin	*Serratia marcescens*
	Fatty acids (corynomycolic acids, spiculisporic acids)	*Corynebacterium lepus*
		Arthrobacter parafineus
		Penicillium spiculisporum
		Talaromyces trachyspermus
	Sulfonylipids	*Capnocytophaga* spp.
	Diglycosyl diglycerides	*Lactobacillus fermentii*
High molecular weight	Alasan	*Acinetobacter radioresistens*
	Emulsan	*Acinetobacter calcoaceticus*
	Biodispersan	*Acinetobacter calcoaceticus*
	Liposan	*Candida lipolytica*
	Mannan-lipoprotein	*Candida tropicalis*
	Food emulsifier	*Candida utilis*
	Insecticide emulsifier	*Pseudomonas tralucida*
	Sulfated polysaccharide	*Halomonas eurihalina*
	Acetyl heteropolysaccharide	*Sphingomonas paucimobilis*

[a] Data are from references 41, 42, 97, 149, 400, and 401.

Several laboratory and field investigations have indicated that the addition of commercial microbial cultures (bioaugmentation) (118, 340, 431, 637) did not significantly enhance rates of oil biodegradation over that achieved by nutrient enrichment (biostimulation) of the natural microbial population (186, 494, 639). The *Exxon Valdez* bioremediation experience, in particular, has been viewed by many as a general rule that bioaugmentation is ineffective in petroleum and other biodegradation processes. This begs two questions: Is there ever a role for inocula in petroleum degradation processes? Is there any potential to exploit recombinant organisms in the practice of environmental bioremediation and waste treatment?

The low water solubilities of the majority of petroleum hydrocarbon compounds have the potential to limit the capacity of microbes, which generally exist in aqueous phases, to access and degrade these substrates. Hydrocarbon-degrading microbes produce a variety of biosurfactants (Table 5) as part of

their cell surface or as molecules released extracellularly (43, 86, 87, 88, 191, 401, 450, 451, 524, 527, 541, 567). These biosurfactants and added chemical surfactants enhance removal of petroleum hydrocarbons from soil or solid surfaces. However, both enhancement and inhibition of biodegradation of hydrocarbons have been observed (35, 356, 618). Suppression of their production, by use of inhibitors or mutagens, retards the ability of these bacteria to degrade oil (41, 491). The low-molecular-weight biosurfactants (glycolipids, lipopeptides) are more effective in lowering the interfacial and surface tensions, whereas the high-molecular-weight biosurfactants (amphipathic polysaccharides, proteins, lipopolysaccharides, and lipoproteins) are effective stabilizers of oil-in-water emulsions (41, 97, 149, 384, 401, 525).

Many studies have characterized the roles of biosurfactants in biodegradation by observing the effects of fractionated preparations (42, 121, 178, 182, 254, 282, 200, 306, 456, 524, 525, 629, 688, 689). However, the successful application of biosurfactants in bioremediation of petroleum pollutants will require precise targeting to the physical and chemical nature of the pollutant-affecting areas. Although many laboratory studies indicate the potential for use of biosurfactants in field conditions, a lot remains to be demonstrated in cost-effective treatment of marine oil spills and petroleum-contaminated soils compared to chemical surfactants.

Chemical surfactants have the ability to emulsify or pseudosolubilize poorly water-soluble compounds thus potentially improving their accessibility to microorgansims. Properties of chemical surfactants that influences their efficacy include charge (nonionic, anionic or cationic), hydrophilic-lipophilic balance (a measure of surfactant lipophilicity), and critical micellar concentration (the concentration at which surface tension reaches a minimum and surfactant monomers aggregate into micelles). Surfactants with hydrophilic-lipophilic balance values from 3 to 6 and 8 to 15 generally promote formation of water-in-oil and oil-in-water emulsions, respectively. Biodegradation of certain poorly soluble petroleum hydrocarbons may be inhibited by surfactants as a result of (i) toxicity by high concentration of surfactant or soluble hydrocarbon; (ii) preferential metabolism of the surfactant itself; (iii) interference with the membrane uptake process; or (iv) reduced bioavailability of miceller hydrocarbons (167, 446, 529).

Typical surfactant concentrations required to wash contaminants out of soil are 1 to 2%, whereas the same contaminants may be solubilized in an aqueous solution at a surfactant concentration of 0.1 to 0.2%. Much of the surfactant added to soil is ineffective as it becomes sorbed to soil particles. Micellarization of the contaminant (at or above the surfactant critical micellar concentration) may prevent access to the contaminant by the microorganism. Diluting the contaminated medium to get the surfactant concentration below its critical micellar concentration can facilitate microbial accession and contaminant degradation (59). When the effects of surfactant physicochemical properties (hydrophilic-lipophilic balance and molecular structure) on the biodegradation of crude oil by a mixed bacterial culture were examined, hydrophilic-lipophilic balance-13 nonylphenolethoxylate substantially enhanced biodegradation at surfactant concentrations of more than critical micellar concentration value (634). Surfactants from other chemical classes with hydrophilic-lipophilic balance values of 13 had no effect or were inhibitory.

The range of stimulatory and inhibitory effects of surfactants on hydrocarbon degradation reported in the literature may not be contradictory but simply describe unique cases based on the nature of the hydrocarbon contaminants, characteristics of the contaminated medium, surfactant properties and the physiology of the organisms involved (260, 631). Understanding how these four elements interact may enable us to design surfactant-enhanced bioremediation systems on a more rational basis (36, 342, 367, 630).

In the following section, the variety of petroleum biodegradation processes will be reviewed, starting with the processes requiring the least microbial expertise and moving on to processes with increasing levels of microbial technological complexity.

Passive bioremediation processes. Natural attenuation, the least invasive approach to bioremediation, requires no intervention other than to demonstrate the progress of the degradation mediated by the indigenous microbial population, and its efficacy remains controversial (270).

Plants and their rhizospheric microorganisms (phytoremediation) can participate in hydrocarbon remediation (47, 151, 162, 262, 402, 419, 422, 498, 536, 549, 595, 650, 678). Plant root exudates can supply carbon and nitrogen sources for microbial growth (12, 486), raising the densities of rhizospheric bacteria by orders of magnitude more than the population in the surrounding soil (12, 138, 536), and enzymes may be produced that degrade organic contaminants (69, 393, 550). Phytoremediation is not a suitable method for remediation of high-volume oily wastes. Volatile organic carbons can be taken up by plants and transpired to the atmosphere without transformation in a process known as phytovolatilization, which is not an acceptable environmental solution. There is limited plant uptake of more hydrophobic and larger petroleum components.

Wetland use in the petroleum industry for removal of inorganic and organic contaminants and toxicity from hydrocarbon wastes was reviewed by Knight et al. (336). Contaminant removal effectiveness depended more on hydraulic loading and influent concentrations than on internal plant communities and water depth. Often biodegradation is accompanied by other removal mechanisms (535). Aerobic processes generally predominate, and the toxicity of contaminants or metabolites is often a problem. The availibility of fertilizer and oxygen is often rate limiting (240, 264, 383, 442, 561).

In general, therefore, these more passive remediation approaches are unlikely to provide sufficient capacity for remediation of high-volume petroleum wastes with their relatively concentrated hydrocarbon contaminant level (typically 2 to 20%).

Landfarming of oily wastes. While landfarming of refinery and wellhead oily sludges is no longer considered environmentally acceptable, it is still being used as an oily sludge treatment and disposal method in many parts of the world (29, 44, 267). As a starting point, large uncontaminated tracts of land are first deliberately contaminated, followed by bioremediation of the less recalcitrant oil fractions. Large refineries, having capacities of 200,000 to 500,000 barrels per day can produce as much as 10,000 cubic meters of sludge per annum. These landfarming operations can therefore result in tying up large

areas of land which will later have to be decommissioned when more environmentally desirable processes are implemented.

Large quantities of volatile organic carbons present in these wastes, which are hazardous to health and which cause tropospheric ozone production, are typically transferred to the atmosphere rather than biodegraded, facilitated by spraying the waste on the land and then routinely tilling the soil to promote gas transfer. In the *Exxon Valdez* spill in the relatively cold Alaskan climate, 15 to 20% of the oil was reported to be lost to the atmosphere by volatilization (219).

Lack of control over the parameters affecting microbial activity (temperature, pH, moisture, aeration, mixing, and circulation) prolongs treatment time (62, 177, 267, 269, 366, 389, 405, 406, 407, 432). Maximum contaminant degradation occurs in the tilled surface, typically amounting to 10 to 20 cm of depth, although deeper aeration and mixing with ploughing and rotovating equipment has also been effectively implemented. The following examples indicate that typical degradation rates of 0.5 to 1% total petroleum hydrocarbon contents per month may be achieved with landfarming. (i) When refinery soil contaminated with 1.3% oil was treated with nutrients, surfactants, and microbial inoculants and the soil was regularly mixed and aerated with deep tilling equipment at air temperatures of around 25°C, total petroleum hydrocarbon contents were reduced by about 90% in 34 days (170). (ii) Landfarming of soil contaminated with 6% No. 6 fuel oil, with nutrient application, control of moisture, and aeration by ploughing and rotovating, resulted in an 80 to 90% reduction in total petroleum hydrocarbon contents in a 6-month span (196). (iii) Landfarming of kerosene-contaminated soil, depth up to 45 cm, with nutrient application and periodic tilling reduced contaminants from 8,700 ppm to 30 to 3,000 ppm (depending on soil depth) (270). Oxygen availability appeared to be a limitation in this project. (iv) Bosert et al. (65) characterized the fate of hydrocarbons during a laboratory study of oily sludge application to soil, simulating an active petrochemical plant landfarming operation. During intensive landfarming of petroleum waste, a gradual accumulation of petroleum hydrocarbons occurred in the soil over time, amounting to 13.8%, wt/wt. Of the total PAHs applied to the soil in the waste, the percentages remaining at the end of treatment were 1.4, 47.4, 78.5, and 78.3% for the 3-, 4-, 5-, and 6-ringed PAHs, respectively. Residual soil concentrations for pyrene and benzo[*a*]pyrene were 245 and 28 ppm, respectively, representing extents of degradation of 14.4 and 44.4%, respectively. At the end of the treatment period, 53% (155 mg of hydrocarbons per g of soil) of the applied hydrocarbons were removed from the soil, representing a degradation rate of 1%, wt/wt (hydrocarbons/soil) per 2 months.

Because of the trend to ban landfarming of petroleum sludges (175) and because thery are considered hazardous wastes, oil companies are seeking other disposal solutions.

Bioreactor-based processes. Most of the rate-limiting and variability factors observed in landfarming of oily sludges may be eliminated in employing simple bioreactors where optimal performance can be achieved by controlling factors affecting rates and extents of microbial growth and oil transformation (270). Bioreactors can accommodate solids concentrations of 5 to 50% wt/vol. Through break up solid aggregates and dispersion of insoluble substrates, hydrocarbon desorption and contact with the aqueous phase is promoted, resulting in increased biodegradation (119).

Bioreactor-based petroleum sludge degradation processes also allow management of volatile organic carbons. By creating reactor conditions which accelerate the process of bioremediation of volatile organic carbons, the biodegradation process rather than volatilization becomes the dominant volatile organic carbon removal mechanism (388, 632). Retaining the more volatile components, which are generally more biodegradable and more supportive of microbial growth and cell energy, supports degradation of the less volatile components, which may rely on cometabolic processes. In more prolonged hydrocarbon biodegradation processes, for example, landfarming, where volatile materials are lost to the atmosphere, the development of microbes on these substrates, containing the catabolic enzymes with relaxed substrate specificities to transform the more recalcitrant compounds, is not facilitated. Diesel fuel stimulated cometabolic mineralization of benzo[*a*]pyrene in culture and in soil (305, 302). The volatile components also help solubilize the more recalcitrant molecules, making them more bioavailable. The ability of paraffin oil to promote mineralization of pyrene was attributed to its solubilizing action (290).

Examples 1 to 3 below describe bioreactor processes having reactor cycle durations of 1 to 4 months (132, 466). Based on an assumed average total petroleum hydrocarbon contents content of 10% in these processes, average degradation rates ranged from about 0.1% to 0.3% total petroleum hydrocarbon contents per day. Example 1: French Limited, Crosby, Tex., refinery and petrochemical wastes were degraded in a slurry-phase aerated and mixed system (173, 174). The inoculum was indigenous microflora, and a novel mixing/aeration system (the MixFlo system) with pure oxygen rather than air was incorporated. Three hundred thousand tons of tar-like material was remediated in 11 months, with 85% of sludge contaminants being destroyed in 122 days. Example 2: Gulf Coast Refinery, a 1-million-gallon bioreactor was used to treat petroleum-impounded sludges (132). The inoculum was hydrocarbon-degrading organisms from a refinery wastewater activated sludge system. Aeration/mixing was done with float-mounted mixer/aerators. Other operating parameters were an average temperature of 22.6°C and nominal solids contents in the reactor of about 10%. A 50% reduction in oil and grease was obtained in 80 to 90 days. The extent of removal of PAHs was 90%. Example 3: Sugar Creek, Mo., refinery sludge treatment in a 5-million-gallon unlined reactor. The inoculum was activated sludge and prepared hydrocarbon cultures. A float-mounted aeration and mixing system was used. Oil and grease concentrations were reduced by 66% (<60 to 90 days), after which the solids were land applied to reduce residual PAHs to below 160 mg/kg (15).

Example 4: the petrozyme process utilizes a well-acclimated culture (see below), an optimized nutrient formulation, a surfactant, and implementing the process in an optimal temperature and pH range, a highly efficient petroleum sludge degradation process was shown to operate in a much shorter cycle time (571, 654). This process, employing eight bioreactors with a total capacity of 1.2 million liters, has been successfully operated for treatment of sludges produced from about 75% of Venezuela's refining capacity. The process has also been im-

plemented at a small number of refineries in the United States, Canada, and Mexico and typically degrades sludges having total petroleum hydrocarbon contents (total petroleum hydrocarbon contents) of 10% wt/vol. Average degradation rates were close to 1% of total petroleum hydrocarbon contents per day. For the initial batch, a mixed microbial culture, acclimated by weekly subculture on crude oil, was used as the inoculum. For each subsequent batch cycle, inoculation is achieved by carryover of a culture fraction from the previous batch. A sparged air-lift aeration system with no mechanical mixing was used. Nutrients and surfactant were formulated to maximize hydrocarbon accession to the microorganisms, microbial growth rates, and rates and extents of hydrocarbon degradation. The operating temperature (28 to 32°C) is maintained without temperature control in the stable Venezuelan climate. pH is maintained in the range from 6.4 to 7.6. The residence time was 10 to 12 days; the extent of degradation of total petroleum hydrocarbon contents was 97 to 99%; and residual PAHs comply with Environmental Protection Agency nonhazardous toxicity characteristics leaching procedure criteria. The process has operated consistently over hundreds of runs at pilot and full scale.

Recent progress in microbiology, molecular biology, and genetics is providing the driving force toward engineering improved biocatalysts (microbes and enzymes) for bioremediation (113, 483, 612). It also offers new tools to collect information on microbial populations in contaminated sites to aid in the evaluation and formulation of strategies for effective bioremediation (655). Risk-based regulatory approaches have continued to put stricter regulation on the field applications of genetically engineered microorganisms (154, 425).

The first demonstration of field release of a genetically engineered microorganism for bioremediation purposes involved use of the engineered strain *Pseudomonas fluorescens* HK44, containing naphthalene catabolic plasmid pUTK21 and a transposon-based bioluminescence-producing *lux* gene fused within a promoter for the naphthalene catabolic genes (515, 544). The environmental release occurred in six lysimeter structures containing soil with and without contaminant PAHs. Soil PAH concentrations were heterogenously dispersed, spatially ranging from 0.04 to 192 ppm. Consequently, a precise evaluation of the effectiveness of *P. fluorescens* could not be adequately determined. However, the concept of developing a genetically engineered strain with the broad metabolic potential required to deal with the diverse array of hydrocarbon components of crude oil has serious shortcomings because of the range of new genes which would need to be incorporated. Moreover, the burden of maintaining all of these genes is likely to make the engineered strains noncompetitive in the natural environment (377).

The above examples describe the performance of microbial processes for degradation of waste hydrocarbons with different levels of process control and optimization. Typical degradation rates of 0.5 to 1% of total petroleum hydrocarbon contents/month obtained in landfarms can be increased to 0.1 to 0.3% per day in simple aerated bioreactor-based processes. With further control, optimization of media and culture conditions, including use of surfactants, degradation rates of 1% per day can be achieved. More-contained bioreactor-based systems also facilitate volatile organic carbon retention. Extents of degradation in landfarming operations are poor, and very limited degradation of higher-molecular-weight PAHs was observed. In contrast, in optimized bioreactor biodegradation systems, overall degradation extents are very high, with reduction of PAHs to below nonhazardous criteria.

The diversity of metabolic pathways required to degrade the range of components in crude oil wastes is likely best provided by a mixed culture suitably acclimated on this substrate. It is unlikely that genetically engineered organisms can contribute to improving the best processes described above, even allowing for the remote possibility that such engineered strains could be used without taking prohibitive cost containment measures, because of the additional costs associated with maintaining a sterile environment excluding competing strains. Further expenses may be incurred if there are restrictions on release of the recombinant strain into the environment.

Biofiltration of Volatile Organic Compounds

Two general types of biofilters exist: solid phase-gas phase biofilters and liquid phase-gas phase biofilters. The biofilter must be amended with appropriate nitrogen, phosphorus, and other nutrients. Inoculation may be used to shorten the start-up or acclimation period (3, 209, 155, 403, 296, 636, 691).

A recurring theme in processes discussed above is that large volumes of volatile components are often transferred to the atmosphere rather than being biodegraded. Petroleum-originating volatile organic carbons, especially BTEX compounds (benezene, tolene, ethylbenzene, *o*-xylene, *m*-xylene,and *p*-xylene), are classified as hazardous environmental priority pollutants. A number of well-established physical separation or destruction technologies exist for controlling the air emission of volatile organic carbons. However, stricter environmental regulations, high costs, and low public acceptance are driving the quest for dependable cost-effective methods for volatile organic carbon treatment, and biological methods are accepted as the most cost competitive.

Biological oxidation of volatile organic carbon vapors by microorganisms immobilized on a solid support material as biofilms and placed in reactors called biofilters provides an effective and inexpensive alternative for removal of volatile organic carbons (40, 134, 376). In these systems, the volatile organic carbon-containing gas phase passes through the high surface solid support phase containing microbial films with little resistance. The media sorb contaminants from the vapor stream and may supply organic and/or inorganic compounds for microbial growth and metabolism (140, 392).

In liquid-gas phase biofilters, the volatile organic carbon-contaminated gas may be sparged or bubbled through a liquid phase. A silicone membrane bioreactor system allowed rapid diffusion of volatile organic carbons and oxygen for the biodegradation of BTEX vapors (34). The system removed BTEX at rates of up to 30 $\mu g\ h^{-1}\ cm^{-2}$ of membrane area, with removal effciencies ranging from 75% to 99% depending on the BTEX concentration and vapor flow rate. Other approaches achieved high gas transfer through use of fine bubble diffusers.

In order to keep the size of the biofilter in realistic proportion, contact or retention time for the gas stream in the biofilter has to be on the order of 30 to 90 s while achieving high

volatile organic carbon removal rates (typically 90%). Consequently, the biofilter must provide the conditions capable of maintaining a microbial population which can support these ambitious objectives. Biofilter microbial activity needs to be able to operate at gas flow rates of around 1 to 2 liters of gas per liter of biofilter capacity per min and degrade around 1 to 2 kg of volatile organic carbons per 1,000 liters of biofilter capacity per day (0.1 to 0.2% per day), which is only a little less than the performance quoted for optimized accelerated petroleum waste bioreactors (676).

Volatile organic carbon biofilters have to be very efficient high-density microbial systems capable of high rates of volatile organic carbon transformation. Optimized gas transfer from the mobile gas phase must be promoted by maximizing the surface area of the solid-phase biofilm or the gas-liquid interfacial area, where the stationary phases are solid and liquid, respectively. Mass transfer of the volatile organic carbons to the degrading microorganisms is particularly challenging because of their hydrophobic nature. Surfactants may be used to promote solubilization of the volatile organic carbons in the aqueous medium or at the solid surface and to increase transfer of the volatile organic carbons from the moblie gas phase (307).

Removal of H_2S and SO_X

High quantities of H_2S and sulfoxides (SO_X) produced in various petrochemical gas and liquid waste streams require treatment, and bacterial processes which purify these streams and convert these by-products to elemental sulfur are now being commercialized. The Thiopaq process ($H_2S + [1/2]\ O_2 \rightarrow S^0 + H_2O$) is a desulfurization process for the production of elemental sulfur from H_2S-containing gas streams by sulfur-oxidizing bacteria (24, 471). Gas streams are first scrubbed with an aqueous washing liquid, with dissolution of the sulfur components into an aqueous phase ($H_2S + OH \rightarrow HS^- + H_2O$). Sulfide-oxidizing thiobacilli, *Thiocalovibrio* and *Thioalcalobacteria* species, convert the sulfides to elemental sulfur ($HS^- + [1/2]\ O_2 \rightarrow S^0 + OH^-$) in the presence of an electron acceptor at neutral pH (85, 284). The bacteria deposit the elemental sulfur outside the cell. The sulfur is separated in a sulfur separator, and the percolation water is recycled to the scrubber. pH- and redox-controlled bioreactors convert as much as 96% of the H_2S, which may be recovered as elemental sulfur and can be removed by available separation methods (620).

These processes with well-known sulfur-oxidizing strains and pathways have only recently been introduced. Environmental parameters are controlled to maximize the long-term process implementation. Technical and commercial efficacy will be determined in the coming years.

Sulfate-rich seawater, commonly injected into the oil reservoirs to enhance secondary oil recovery, may stimulate the growth of sulfate-reducing bacteria in the reservoirs, with subsequent H_2S production. This biogenic H_2S production, also known as reservoir souring, is of major concern to the oil industry. H_2S is corrosive, increases sulfur content in oil and gas, and may also lead to reservoir plugging (250). Reduction in H_2S formation by addition of nitrate to the injection water has been reported (508, 607). The beneficial effect of nitrate injection for stimulation of a competing group of nitrate-reducing bacteria has been demonstrated in several model experiments (447, 508) and successful field applications (288, 607). Following nitrate injection, nitrite inhibition of sulfate-reducing bacteria and sulfide oxidation by nitrate-reducing bacteria have been suggested as the mechanisms for H_2S elimination (288, 447). Nitrite reductase-containing sulfate-reducing bacteria can overcome this inhibition by further reducing nitrite to ammonia (225). Nitrite reductase can be regarded as a resistance factor that prevents the inhibition of dissimilatory sulfate reduction by nitrite.

MICROBIAL PROCESSES FOR RECOVERING AND UPGRADING PETROLEUM

Microbial Enhanced Oil Recovery

In microbial enhanced oil recovery processes, microbial technology is exploited in oil reservoirs to improve recovery (41, 122, 589). From a microbiologist's perspective, microbial enhanced oil recovery processes are somewhat akin to in situ bioremediation processes. Injected nutrients, together with indigenous or added microbes, promote in situ microbial growth and/or generation of products which mobilize additional oil and move it to producing wells through reservoir repressurization, interfacial tension/oil viscosity reduction, and selective plugging of the most permeable zones (81, 82). Alternatively, the oil-mobilizing microbial products may be produced by fermentation and injected into the reservoir.

This technology requires consideration of the physicochemical properties of the reservoir in terms of salinity, pH, temperature, pressure, and nutrient availability (319, 320). Only bacteria are considered promising candidates for microbial enhanced oil recovery. Molds, yeasts, algae, and protozoa are not suitable due to their size or inability to grow under the conditions present in reservoirs. Many petroleum reservoirs have high NaCl concentrations (286) and require the use of bacteria which can tolerate these conditions (558). Bacteria producing biosurfactants and polymers can grow at NaCl concentrations of up to 8% and selectively plug sandstone to create a biowall to recover additional oil (499).

One microbial enhanced oil recovery approach successively limits the carbon sources and increases the temperature, pressure, and salinity of the media to select microbial strains capable of growing on crude oil at 70 to 90°C, 2,000 to 2,500 lb/in^2, and a salinity range of 1.3 to 2.5% (27). Thermophilic isolates potentially useful for microbial enhanced oil recovery have been described (14, 395). Extremely thermophilic anaerobes that grow at 80 to 110°C have been isolated and cultured in the laboratory. All of these organisms belonged to the arachaebacteria, living autotrophically on sulfur, hydrogen, and carbon dioxide by methanogenesis and heterotrophically on organic substrates by sulfur respiration or anaerobic fermentation.

A one-dimensional model was developed to simulate the microbial enhanced oil recovery process (150). The model involved five components (oil, bacteria, water, nutrients, and metabolites), with adsorption, diffusion, chemotaxis, growth and decay of bacteria, nutrient consumption, permeability damage, and porosity reduction effects. Comparison between the experimental and simulated results emphasized the validity

TABLE 6. Microbial products and their applications in enhanced oil recovery[a]

Product	Microorganism	Application in oil recovery
Biomass	*Bacillus licheniformis* *Leuconostoc mesenteroides* *Xanthomonas campestris*	Selective biomass plugging Viscosity reduction Oil degradation, wetability alteration
Biosurfactants (emulsan, sophorolipids, peptidolipid, rhamnolipid)	*Acinetobacter calcoaceticus* *Arthrobacter paraffineus* *Bacillus licheniformis* *Clostridium pasteurianum* *Corynebacterium fasciens* *Pseudomonas rubescens*	Emulsification, decrease of interfacial tension, viscosity reduction
Biopolymers (alginate, xanthan, dextran, pullulan)	*Bacillus polymyxa* *Brevibacterium viscogenes* *Leuconostoc mesenteroides* *Xanthomonas campestris*	Injectivity profile modification, mobility control
Solvents (*n*-butanol, acetone, ethanol)	*Clostridium acetobutylicum* *Clostridium pasteurianum* *Zymomonas mobilis*	Oil dissolution, viscosity reduction
Acids (acetate, butyrate)	*Clostridium* spp. *Enterobacter aerogenes*	Permeability increase, emulsification
Gases (CO_2, CH_4, H_2)	*Clostridium acetobutylicum* *Clostridium acetobutylicum* *Enterobacter aerogenes* *Methanobacterium* sp.	Increased pressure, oil swelling, decrease of interfacial tension, viscosity reduction, permeability increase

[a] Data are from references 41, 195, 499, 558, and 584.

of the simulator developed and determined its degree of accuracy (average absolute relative error, 8.323%). Oil recovery was found to be sensitive to variations in the concentration of injected bacteria, the size of the bacterial culture plug, incubation time, and residual oil saturation.

Microbial enhanced oil recovery-participating microorganisms produce a variety of fermentation products, e.g., carbon dioxide, methane, hydrogen, biosurfactants, and polysaccharides from crude oil, pure hydrocarbons, and a variety of nonhydrocarbon substrates (Table 6). Xanthan gum, a microbial biopolymer, is frequently used in microbial enhanced oil recovery field testing (195, 558), often with base-hydrolyzed polyacrylamide as a copolymer. Desirable properties of polymers for microbial enhanced oil recovery include shear stability, high solution viscosity, compatibility with reservoir brine, stable viscosity over a wide range of pH, temperature, and pressure, and resistance to biodegradation in the reservoir environment (195, 286, 539). Organic acids produced through fermentation readily dissolve carbonates and can greatly enhance permeability in limestone reservoirs, and attempts have been made to promote their anaerobic production (589). Organic solvents and dissolved CO_2 can decrease oil viscosity. Fermentation gases can repressurize wells, leading to displacement and production of light or conventional crude oil through a revitalized gas-driven mechanism (589).

Residual oil in reservoirs can be recovered when highly permeable watered-out regions of oil reservoirs are plugged with bacterial cells and biopolymers (584). Bacteria and nutrients are injected into the reservoir, and the system is shut in to allow the biomass to plug the more permeable region as it grows (280, 585). Water is then injected (water flooding) to force oil trapped in less permeable regions of the reservoir out into the recovery well. A porous glass micromodel has been used to simulate biomass plugging with *Leuconostoc mesenteroides* under nutrient-rich conditions (329, 360, 584, 585, 671). As nutrients flow through the porous glass, a biomass plug establishes at the nutrient-inoculum interface. High substrate loading and high pH promoted plug development (671). The residual oil remaining after water flooding is a potenial target for selective reservoir plugging of porous rocks with in situ bacterial growth on injected nutrients (195, 289). Bacteria may exert a much greater plugging effect when they multiply within the reservoir rock rather than when they are injected and accumulate at the surface.

Added or in situ-produced biosurfactants, which aid oil emulsification and detachment of oil films from rocks, have considerable potential in microbial enhanced oil recovery processes (41, 42). Emulsan reduced the viscosity of Boscon heavy crude oil from 200,000 cP to 100 cP, facilitating heavy oil pumping (246). Biosurfactant from the thermo- and halotolerant species, *Bacillus licheniformis* isolates and thermotolerant *Bacillus subtilis* strains have been tested for with various levels of success in reservoirs and in laboratory simulations (285, 385, 400, 674, 675).

In a field microbial enhanced oil recovery study in the Southeast Vassar Vertz Sand Unit salt-containing reservoir in Oklahoma, nutrient injection stimulated growth of the microbial populations, including several aerobic and anaerobic heterotrophic bacteria, sulfate-reducing bacteria, and methanogenic halophiles. Nutrient-stimulated microbial growth produced a 33% drop in the effective permeability in an injection well at North Burbank Unit in Oklahoma, plugging off high-perme-

TABLE 7. Potential microorganisms with petroleum deemulsification properties

Microrganism	Petroleum oil emulsion tested	Emulsion type	Reference(s)
Acinetobacter calcoaceticus	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	449
Acinetobacter radioresistans	Kerosene-water model	Water-in-oil	449
Aeromonas sp.	Kerosene-water model	Oil-in-water	455
Alteromonas sp.	Kerosene-water model	Oil-in-water	455
Alcaligenes latus	Kerosene-water model	Water-in-oil	449
Corynebacterium petrophilum	Kerosene-water model; crude oil-water	Water-in-oil	161, 583
Bacillus subtilis	Crude oil-water model	Oil-in-water	283
Micrococcus sp.	Kerosene-water	Oil-in-water; water-in-oil	141
Nocardia amarae	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	95, 346
Pseudomonas carboxydohydrogena	Kerosene-water model	Water-in-oil; oil-in-water	449
Rhodococcus aurantiacus	Kerosene-water model	Water-in-oil; oil-in-water	503
Rhodococcus rhodochrous	Kerosene-water model	Water-in-oil; oil-in-water	667
Rhodococcus rubropertinctus	Kerosene-water model	Water-in-oil; oil-in-water	345
Torulopsis bombicola	Oilfield emulsions	Water-in-oil	161
Mixed bacterial culture	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	448

ability layers and diverting injection fluid to zones of lower permeability and higher oil saturation (287). In contrast to the poor experience with exogenous organisms for bioremediation (bioaugmentation), injection of selected microbial species into oil field plots in Japan and China resulted in improved oil recoveries of 15 to 23% (248, 680). In one case microbial treatment caused some degradation of long-chain aliphatic hydrocarbon chains but with no apparent degradation of aromatic ring structures.

More than 400 microbial enhanced oil recovery field tests have been conducted in the United States alone, mostly as single-well stimulation treatments on low-productivity wells, so that reliable data are sparse (319, 320, 589). Reservoir heterogeneity significantly affects oil recovery efficency. Microbial enhanced oil recovery technology may be attractive to independent oil producers, who mostly operate "stripper wells" (producing an average of 0.2 to 0.4 ton of oil per day), of which there are about 470,000 in the United States. A single-well stimulation treatment might increase the rate of production from 0.2 to 0.4 ton of oil per day and sustain the increased rate for 2 to 6 months without additional treatments.

The microbial enhanced oil recovery process may modify the immediate reservoir environment in a number of ways that could also damage the production hardware or the formation itself (280). Certain sulfate reducers can produce H_2S, which can corrode pipeline and other components of the recovery equipement.

Despite numerous microbial enhanced oil recovery tests, considerable uncertainty remains regarding process performance. Ensuring success requires an ability to manipulate environmental conditions to promote growth and/or product formation by the participating microorganisms. Exerting such control over the microbial system in the subsurface is itself a serious challenge. In addition, conditions vary from reservoir to reservoir, which calls for reservoir-specific customization of the microbial enhanced oil recovery process, and this alone has the potential to undermine microbial process economic viability.

Microbial enhanced oil recovery systems currently represent high-risk processes to oil producers looking for efficient and predictable oil recovery. Modeling approaches which can simulate reservoir conditions and facilitate the development of more reliable oil recovery strategies may represent a small but uncertain ray of hope, but progress in this area is slow. Development of a universal additive mixture, consisting of a combination of microbial strains, nutrients, surfactants, and buffering agents in appropriate proportions, may represent a further productive line of research.

Microbial Deemulsification

Oilfield water-in-oil emulsions, formed at various stages of exploration, production, and oil recovery, represent a major problem for the petroleum industry (48, 362, 404, 551). These emulsions are characterized according to their stability as tight (microemulsion, very fine droplets of around 100 Å, hard to break) or loose (coarse droplets, size around 5 μm, unstable, easily broken) (48, 362). Water and dirt in crude oil cause corrosion and scaling on pipelines and reactors, and a maximum sediment and water content of 0.5 to 2.0% is required for pipeline-quality oil (375, 580). To produce saleable oil, petroleum water-in-oil emulsions must be destabilized by costly physical and/or chemical methods.

Microbial species including *Nocardia amarae* (95), *Corynebacterium petrophilum* (583), *Rhodococcus auranticus* (503), *Bacillus subtilis* (283), *Micrococcus* spp. (141), *Torulopsis bombicola* (161), and *Pseudomonas*- and *Acinetobacter*-containing mixed bacterial cultures (448, 449, 653) exhibited deemulsification capabilities (Table 7). Microorganisms generally exploit petroleum hydrocarbon-induced hydrophobic cell surfaces or hydrophobic/hydrophilic properties of biosurfactants to displace or alter the emulsifiers that are present at the oil-water interface (41, 345, 346, 449), although some organisms grown on nonpetroleum hydrocarbon substrates also deemulsified petroleum emulsions (161, 283, 343). Some biologically produced agents such as acetoin (283), polysaccharides, glycolipids, glycoproteins, phospholipids, and rhamnolipids (345) destabilized petroleum emulsions. Surfaces of bacterial cells were responsible for the major deemulsifying activity of *Nocardia amarae* (346) and the mixed bacterial culture (448, 449).

In pure-culture deemulsification studies with pure bacterial cultures, the relationship between initial rate of deemulsification and cell concentration was linear, while that between the extent of deemulsification and cell concentration was logarith-

mic (95, 141, 448). A positive correlation was observed between cell concentration and rate of deemulsification by *C. petrophilum* (161) and *Micrococcus* spp. (141).

Emulsion-breaking activity was not affected by lyophilization or freezing/thawing, but was destroyed by autoclaving (448), whereas the deemulsifying properties of *N. amarae*, *R. auran-tiacus*, and *R. rubropertinctus* were resistant to autoclaving (344, 345). Alkaline methanolysis destroyed bacterial cell deemulsification ability (345). Washing the cells with any lipid-solubilizing solvent yielded a decrease in their deemulsification cability for water-in-oil emulsions.

The microbial deemulsification rate varies with differences in emulsion composition. Pure cultures of *N. amarae*, *C. petrophilum*, and the yeast *T. bombicola* deemulsified water-in-oil petroleum emulsions diluted with toluene (161, 583). The high viscosity of the emulsion prevented pure bacterial isolates from causing significant deemulsification by *N. amarae* or *R. rhodochrous* (667). Elevating the temperature, which reduces apparent viscosity, generally accelerates deemulsification (344, 430). Microbial deemulsification with a mixed bacterial culture was highest at 50°C (449).

The above discussion raises the question of how some microbial species known to produce biosurfactants and promote petroleum emulsion formation and also some bioemulsifiers, such as rhamnolipids, participate in deemulsification. While the processes involved are undoubtedly complex, microbial deemulsifying activity has generally been observed in water-in-oil emulsions, whereas microbial bioemulsification processes occur during microbial oil biodegradation in oil-in-water emulsions. These are very different physical states, as demonstrated by the fact that chemical surfactants which stabilize oil-in-water emulsions are not effective in stabilizing water-in-oil emulsions and vice versa. Indeed surfactants effective in stabilizing oil-in-water and water-in-oil emulsions have different hydrophilic-lipophilic balances.

Deemulsification of water-in-oil emulsions requires the hydrophilic cell surfaces which exist around cells growing exponentially and in early stationary phase, whereas deemulsification of oil-in-water emulsions requires hydrophobic surfaces produced during the endogenous metabolic phase (345). This suggests that different physiological properties support deemulsification of oil-in-water and water-in-oil emulsions. Additionally, emulsification in a continuous aqueous phase (oil-in-water) is very much a dynamic aerobic microbial growth process. In contrast, in a continuous oil phase (water-in-oil), the low oxygen transfer to microbial cells concentrated in aqueous droplets will limit microbial growth. Any deemulsification effect will likely be due to the predominantly nongrowing cells which were added as an inoculum to the system.

Generally, physicochemical deemulsification processes are capital intensive, and emulsions often generated at the wellhead have to be transported to central processing facilities. Because of the characteristic ability of microorganisms to exert their effects at nonextreme conditions, an effective microbial deemulsifier could be used directly to treat emulsions at the wellhead, thus saving on transport and high capital equipment costs. However, due to the great variability among the properties of crude oil emulsions, inconsistencies are experienced in the performance of all deemulsification processes, physical, chemical, and biological. Further research on microbial deemulsification processes needs to be aimed at the development of more reliable and universally effective systems.

Microbial Desulfurization

Sulfur is usually the third most abundant element in crude oil, normally accounting for 0.05 to 5%, but up to 14%in heavier oils (580, 139, 610). Most of the sulfur in crude oil is organically bound, mainly in the form of condensed thiophenes, and refiners use expensive physicochemical methods, including hydrodesulfurization to remove sulfur from crude oil (557). These high costs are driving the search for more efficient desulfurization methods, including biodesulfurization (201, 387, 554). In developing a lower cost biologically based desulfurization alternative, promoting selective metabolism of the sulfur component (attacking the C-S bonds) without simultaneously degrading the nonsulfur (C-C bonds) fuel components in organic sulfur will be the most important consideration (201, 352).

Aerobically grown strains, such as *Rhodococcus erythropolis* and related species, remove the sulfur from compounds such as dibenzothiophene (DBT) without degrading the carbon ring structure (325). These strains can use sulfur from DBT as a sole source of sulfur, which facilitates a strategy for isolation of desulfurizing organisms. Other aerobic selective desulfurizing microbes include *Nocardia* spp., *Agrobacterium* sp. strain MC501 (130), *Mycobacterium* spp. (452), *Gordona* sp. strain CYKS1 (218), *Klebsiella* spp. (157), *Xanthomonas* spp. (131), and the thermophile *Paenibacillus* (341).

Rhodococcus sp. strain IGTS8 was isolated from a mixed culture obtained from a sulfur-limited continous-culture system capable of using organically bound sulfur (293, 434, 469). Strain IGTS8 converts DBT to dibenzothiophene-5-oxide (DBTO), then to dibenzene-5,5-dioxide ($DBTO_2$), then to 2-(2-hydroxybiphenyl)-benzenesulfinate (HPBS), and finally to 2-hydroxybiphenyl (HBP) to release inorganic sulfur (464, 465) in a pathway involving two monooxygenases and a desulfinase (224). This enzyme system also transforms alkyl- and aryl-substituted DBT (373). Since the HBP product partitions into the oil phase, its fuel value is not lost. The flammability and explosive risks from the above oxygen-requiring process have led to consideration of cloning the desulfurization genes into anaerobic hosts, which would hyperproduce the enzymes for addition to the crude oil. Desulfurization rates for nonengineered *Rhodococcus* spp. are 1 to 5 mg of HBP per g of dry cells per h, with 55 to 75% of the DBT being released as HBP (309).

Strain IGTS8 exhibits little activity towards thiophenes and benzothiophenes, so new biocatalysts with broad substrate specificity need to be engineered (32). Improved biocatalysts have been engineered, and the desulfurization genes have been manipulated (224, 416, 469, 500, 562). The desulfurization genes of IGTS8 have been characterized, and directed evolution and gene shuffling approaches have broadened their substrate specificity. Strains with deletions of the gene encoding dibenzothiophene sulfone monooxygenase (DszA) or hydroxyphenyl benzene sulfinase (DszB) in the biodesulfurization pathway (Fig. 4) have been prepared, allowing possible production of potentially valuable sulfur-containing metabolic intermediates as products. Thus, new biocatalysts lacking DszB

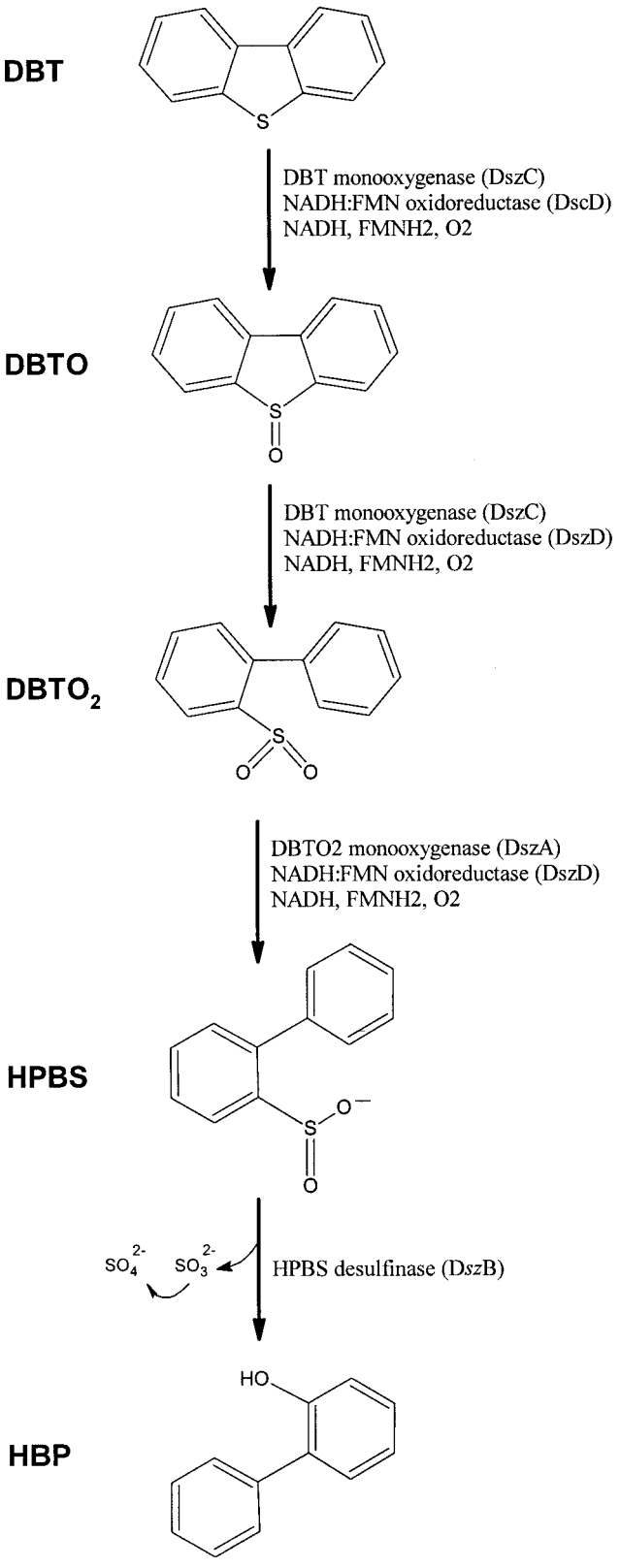

FIG. 4. Proposed sulfur-specific pathway for dibenzothiophene (DBT) desulfurization by *Rhodococcus* species. Abbreviations: DBTO, dibenzothiophene sulfoxide; $DBTO_2$, dibenzothiophene sulfone; HPBS, hydrophenyl benzene sulfinate; HBP, hydroxy biphenyl. The *Rhodococcus* pathway does not continue to intermediary metabolism and stops with the release of hydroxy biphenyl, and therefore no decrease in carbon content occurs (435). The physiological significance of the pathway is to obtain sulfur for growth. DszA, DszB, DszC, and DszD are the catalytic gene products of *dszA*, *dszB*, *dszC*, and *dszD*, respectively.

or DszA stopped desulfurization at the sulfinate or sulfone step for the generation of saleable products with higher desulfurization rates (435). The recombinant *Rhodococcus* sp. strain T09, constructed with a *Rhodococcus-Escherichia coli* shuttle vector, utilized both DBT and benzothiophene as the sole sulfur source (413). The recombinant cells were able to desulfurize alkylated DBT and benzothiophene and also alkylated DBT in an oil-water, two-phase resting cell reaction.

The general water needs of microbial cells require the creation of a two-phase biodesulfurization system with high interfacial areas through energy-intensive mixing and/or addition of a surfactant, with a postdesulfurization deemulsification step. Defining a cost-effective two-phase bioreactor system with subsequent oil-water separation and product recovery represents a key challenge to the viability of biodesulfurization processes (435). Multiple-stage air-lift reactors reduced mixing costs and promoted mass transfer (469), while centrifugal methods were effectively used to break the emulsion, recover the desulfurized oil, and recycle the cells (682).

Since the oxygen-requiring desulfurization enzymes in *Rhodococcus* sp. strain IGTS8 are associated with the external hydrophobic membrane surfaces (148, 311, 459), it has been hypothesized that the enzymes should be active in nonaqueous media. The maximum oil-water ratio for desulfurization was found to be 1.25 ml/g. However 82% sulfur removal was obtained at a 9:1 oil-water ratio (476). Surfactants stimulated biodesulfurization in oil-water systems (476).

Critical aspects of the biodesulfurization process development include reactor design, product or by-product recovery, and oil-water separation. New concepts include the use of multistaged air-lift reactors to reduce the cost of mixing and overcome poor reaction kinetics and to achieve continuous growth and regeneration of the biocatalyst in the same system rather than in a separate reactor (434). Tight emulsions, formed by good oil-cell-water contact and mixing, can be separated continuously with hydrocyclones to obtain relatively clean oil and water. Compared to aqueous systems, biodesulfurizations carried out in two-phase aqueous-alkane solvent systems (309, 460, 461, 469) exhibited increased sulfur removal rates. The extent of biodesulfurization varied with the nature of the oil feedstock, ranging from around 20 to 60% for crude oil and light gas oil (109, 469, 488) to 30 to 70%, 40 to 90%, 65 to 70%, and 75 to 90% for middle distillates, diesel, hydrotreated diesel, and cracked stocks, respectively (469, 488). Hence, the problems of creating two-phase oil-water systems for biodesulfurization of viscous crude oils are circumvented by using more refined products, such as diesel or gasoline (436).

The 1990 Clean Air Act Amendment set the sulfur content of diesel fuel at a maximum of 500 ppm (623), but future values for diesel fuel may be as low as 30 ppm (624). Existing microbial desulfurization technology is not cost effective for heavy or middle distillates of crude oil (416), and hydrodesulfurization technologies cannot achieve the 30-ppm levels required in the

TABLE 8. Microorganisms with potential petroleum-biorefining activities

Biorefining process	Biocatalyst	Microorganism	Reference(s)
Desulfurization	Aerobic bacteria	*Rhodococcus erythropolis* H2	461
		Arthrobacter sp.	372
		Corynebacterium sp. strain SY1	465
		Nocardia sp.	434
		Agrobacterium sp. strain MC501	130
		Mycobacterium sp. strain G3	452
		Gordona sp. strain CYKS1	218
		Klebsiella sp.	157
		Paenibacillus sp.	341
		Pseudomonas alcaligenes	242
		Rhodococcus sp. strain IGTS8	293
		Rhodococcus sp. strain ECRD-1	229
		Xanthomonas sp.	131
	Anaerobic bacteria	*Desulfovibrio desulfuricans* M6	328, 331
Denitrogenation	Aerobic bacteria	*Pseudomonas ayucida* IGTN9m	326
		Pseudomonas aeruginosa	6
		Pseudomonas sp. strain CA10	542, 543
		Pseudomonas putida 86	480
		Pseudomonas stutzeri	563
		Rhodococcus sp. strain B1	480
		Comamonas acidovorans	619
		Comamonas testosteroni	546
		Nocardioides sp.	511
Demetalation	Chloroperoxidase	*Caldariomyces fumago*	188, 429
	Cytochrome *c* reductase; heme oxygenase	*Bacillus megaterium*, *Escherichia coli*	673

future. However, a combination of biodesulfurization and hydrodesulfurization technology has the potential to achieve these levels (229).

The activities of key enzymes in the desulfurization pathway have also been increased 200-fold (223, 224, 469, 500). A preliminary process design aimed at reducing the sulfur content of gasoline from 1,000 ppm to 100 ppm has been described (622). Gasoline-tolerant bacteria containing the desulfurizing enzyme are available. Any process for gasoline biodesulfurization must achieve costs below the predicted 1.5 cents/gallon cost estimate for alternative innovative chemical-physical desulfurization processes (32).

Microbial Denitrogenation

Crude oil contains about 0.5 to 2.1% nitrogen, with 70 to 75% consisting of pyrroles, indoles, and carbazole nonbasic compounds. Carbazole is a potent inhibitor of hydrodesulfurization, poisons cracking catalysts, is both toxic and mutagenic, and contributes to the formation of undesirable air-polluting nitric oxides (55, 580). Nitrogenous compounds are generally eliminated from petroleum by expensive hydrotreatment under high temperatures and pressures.

Several species of bacteria that can utilize indole, pyridine, quinoline, and carbazole and its alkyl derivatives have been isolated and characterized, including *Alcaligenes*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Comamonas*, *Mycobacterium*, *Pseudomonas*, *Serratia*, and *Xanthomonas* (6, 66, 190, 222, 299, 311, 457, 480, 511, 563, 619). Bacteria exhibit some general similarities in the pathways for the transformation of aromatic compounds. Oxygenases play an important role in the initial attack in the transformation of nitrogen compounds (187, 299, 545, 546). The initial enzymatic conversion steps yield dihydroxylated intermediates, which then follow either a *meta*- or an *ortho*-pathway, leading to intermediates of central metabolic pathways. Pyrrole and indole are easily degradable, but carbazole is relatively resistant to microbial attack. Recently, selective removal of nitrogen from quinoline by *Pseudomonas ayucida* IGTN9m was reported (326). Increasingly stringent regulations on the nitrogen content of fossil fuels will require very low levels of these heteroaromatic compounds.

Sato et al. (542, 543) identified and cloned the genes responsible for carbazole degradation by *Pseudomonas* sp. strain CA10. To investigate the substrate specificity of the *carA* gene product, a plasmid bearing the *carAa*, *carAc*, and *carAd* genes and expressing only *carA*-encoded proteins was constructed. When introduced into *E. coli*, the recombinant strain was able to transform a wide range of aromatic compounds, including carbazole, *N*-methylcarbazole, *N*-ethylcarbazole, dibenzofuran, dibenzothiophene, dibenzo-*p*-dioxin, fluorene, naphthalene, phenanthrene, anthracene, and fluoranthene.

The major barrier to using a microbial process to remove nitrogen from crude oil is the same as that for desulfurization, namely, the need to create an oil-water two-phase system. Removal of nitrogen and sulfur requires specific attack of the C-N and C-S bonds, respectively, but not C-C bond attack, thus preserving the fuel value of the residual products. To make economic sense, denitrogenation processes need to be integrated with a crude oil desulfurization step (55). However, more recent wisdom has been to retain the hydrodesulfurization technology for initial desulfurization and denitrogenation, with use of microbial desulfurization to further reduce the sulfur level in refined fuels such as diesel and gasoline. Micro-

TABLE 9. Bacterial biosensors for monitoring petroleum contaminants

Bacterial biosensor	Contaminant	Reporter gene fusion	Reference
Pseudomonas fluorescens HK44	Naphthalene	*nahG-luxCDABE*	252
Pseudomonas putida RB1401	Toluene, xylene	*xylR-luxCDABE*	89
Pseudomonas putida B2	BTEX	*tod-luxCDABE*	25
Pseudomonas putida TVA8	BTEX	*tod-luxCDABE*	25
Escherichia coli DH5α	Alkanes	*alkB-luxAB*	586
Escherichia coli DH5α	BTEX	*xylR-luc*	668
Escherichia coli	Benzene derivatives	*xylS-luc*	273

organisms with potential biorefining activtities are shown in Table 8.

Enzymatic Upgrading of Petroleum Fractions and Pure Hydrocarbons

The unique regio- and stereospecificity properties of enzymes combined with their ability to catalyze reactions in nonaqueous media opens up opportunities to exploit enzyme technology in petroleum processing.

Stereoselective biocatalytic hydroxylation reactions, cytochrome p450-dependent monooxygenases, dioxygenases, lipooxygenases, and peroxidases (114, 259) have tremendous potential for enantiospecific conversions involving petrochemical substrates and their derivatives. Naphthalene dioxygenase (NDO) can produce a range of attractive diol precursors for chemical synthesis and also catalyzes a variety of other oxidations, including monohydroxylation, desaturation, O- and N-dealkylation, and sulfoxidation (339). Because of its broad specificity towards a wide range of aromatic hydrocarbons, NDO can produce chiral petrochemical-based precursors for the synthesis of specialty chemicals (70, 216, 509, 510). Chiral cyclohexadiene diols are potential precursors for the enantiospecific synthesis of many bioactive molecules, and toluene dioxygenase has been used for biosynthesis of enantiomers of erythrose (78, 99, 266, 555, 665). *cis*-Chlorodihydrodiol is an extremely versatile synthon (265). Furthermore, NDO and toluene dioxygenase sometimes form opposite enantiomers of the same product from the same substrate (339).

Epoxides are produced by the action of some monooxygenases, especially the cytochrome P450 monooxygenases, as well as in other epoxidations occurring in biosynthetic pathways (1). Chiral alkane epoxides are synthons for a variety of different syntheses. The alkane hydroxylase and xylene oxygenases of *P. putida* are versatile monooxygenases for stereo- and regioselective oxidation of aliphatic and aromatic hydrocarbons (600, 672). Epoxide hydrolases can transform the resulting epoxides into diols (353). The alkane hydroxylase of *P. oleovorans* has broad specificity and can convert a range of alkanes, alkanols, alkanals, alkenes, and other substrates into interesting products in two-phase systems (548, 628). For some systems, bioconversion rates producing chemical products in the cost range of US$3 to US$10 per kg have been predicted (670).

A recombinant *E. coli* strain containing the *P. oleovorans alk* genes was able to grow on sugars in the presence of a bulk *n*-alkane phase and convert octane to the corresponding octanoic acid (184). To overcome degradation of the products of the xylene monooxygenase from *P. putida*, the *xyl* genes from the TOL plasmid encoding this enzyme may be inserted in *E. coli* (672). *P. oleovorans* can convert octane to medium-chain poly(3-hydroxyalkanoates), with potential for use in biodegradable plastics (247), at projected large-scale manufacturing costs of less than US$10 per kg (348).

A number of oxidative enzymes have been the target of directed evolution (115, 116). Cytochrome $P450_{cam}$ monooxygenase from *P. putida* has successfully evolved to function more efficiently in the hydroxylation of naphthalene (516), and dioxygenases with improved thermostability and substrate specificity have been designed (207, 324, 354, 463).

In active hybrids of naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems, replacement of small subunits affected the rate of product formation but had no effect on the substrate range, regiospecificity, or enantiomeric purity of oxidation products with the substrates tested (474). Substitiution of valine or leucine for Phe-352 near the active site iron in the α-subunit of NDO altered the stereochemistry of naphthalene *cis*-dihydrodiol formed from naphthalene and also changed the region of oxidation of biphenyl and phenanthrene (473, 475).

New protein engineering developments will undoubtedly result in the creation of powerful biocatalysts with applications for specific transformations or upgrading of petroleum fractions or pure hydrocarbon compounds. Such developments have already occurred with simpler biocatalytic systems, such as the extracellular microbial enzymes.

However, in general, the use of enzymes in synthesis has to exploit the main competitive advantage of enzyme over chemical methods, namely, for stereo- and regiospecific synthesis, producing single isomeric products. This limits the range of reactions, usually to production of bioactive compounds or precursors, while biocatalytic systems in non-aqueous-phase media has extended the range of substrates accessible to enzymes to include hydrophobic petroleum compounds; reaction rates in non-aqueous-phase media are often much lower than in aqueous systems. These drawbacks limit the applicability of this technology to specialty chemicals and steer it away from bulk petroleum processing.

BACTERIAL BIOSENSORS

Bacterial biosensors uniquely measure the interaction of specific compounds through highly sensitive biorecognition processes and offer great sensitivity and selectivity for the detection and quantification of target compounds (315, 608). Whole-cell biosensors, constructed by fusing a reporter gene to a promoter element induced by the target compound, offer the ability to characterize, identify, quantify, and determine the biodegradabilty of specific contaminants present in a complex mixture without pretreatment of the environmental samples

(11, 142, 179, 483). The genetic information, located on a plasmid vector, is inserted into a bacterial strain so that the engineered fusion replicates along with the cell's normal DNA. Biosensor systems include a wide range of integrated devices that employ enzymes, antibodies, tissues, or living microbes as the biological recognition element. Bacterial biosensors developed for monitoring petroleum contaminants are shown in Table 9.

There is a continuing need to monitor the concentration, transformation, and toxicity of common soil and groundwater pollutants, including petroleum contaminants such as BTEX and PAH compounds in the environment. Many current analytical techniques used for monitoring pollutants require expensive equipment and extensive pretreatment of the environmental samples. The inherent difficulties in classical analytical methods have created an interest in the development of alternative methods, including novel bacterial biosensors. These biosensors offer significant advantages over conventional analytical methods. Classical analytical methods cannot distinguish between unavailable and bioavailable compounds. While conventional analytical methods provide information about concentrations in the contaminated phases, they do not assess the bioavailability of a contaminant, which is an important consideration of site remediation (11). Bacterial biosensor measurements have also been shown to be within very close range of those measured by standard gas chromatography-mass spectroscopy techniques (e.g., 3% in the case of toluene) (668).

The presence of toxic compounds and the potential associated ecological risks can be determined by using bacterial biosensor and toxicity tests. Although several biochemical and genetic methods which give clear signal or bands are available, data on field environmental quality assesment are limited. There are some outstanding questions. Are microbes capable of degrading the particular pollutant present in the contaminated site and will the biological treatment method effectively remove the contaminants? What happens if the concentration of the contaminant is low compared to that of other biodegradable or metabolizable substrates? Although these questions may not be answered, molecular and biochemical tools available today would help provide some of the answers in the coming years.

Broad-specificity biosensensors are used for toxicity testing and respond to a wide range of compounds, including petroleum hydrocarbons in contaminated soils, a good example of which is the commercially available Microtox assay, used for measuring the toxicity of environmental samples by monitoring the light production of the naturally bioluminiscent marine bacterium *Photobacterium phosphoreum* (89). Since bacterial bioluminescence is tied directly to cellular respiration, any inhibition of cellular metabolism due to toxicity results in a decrease in the light emission of the affected cells. In nonspecific bacterial biosensors, *lux* genes are fused to heat shock promoters so that exposure of the cells to toxic organic compounds or metals rapidly induces light production (142).

With *P. fluorescens* HK44, a prototype bioluminescent catabolic reporter strain, a bioassay for the quantitative assessment of naphthalene and salicylate biodegradation in aqueous, soil, and slurry systems is available (252, 253). A linear relationship was established between substrate concentration and bioluminescence over a concentration range of up to two orders of magnitude, and naphthalene induced a significant response at a concentration as low as 45 ppb. The potential use of immobilized *P. fluorescens* HK44 cells for on-line monitoring of PAH degradation in the subsurface has also been demonstrated (657).

A biosensor for detecting the toxicity of PAHs in contaminated soils was constructed with an immobilized recombinant bioluminescent bacterium, GC2 (*lac*::*luxCDABE*), which constitutively produces bioluminescence (231). The monitoring of phenanthrene toxicity was achieved through measurement of the decrease in bioluminescence when a sample extracted with the rhamnolipid biosurfactant was injected into a minibioreactor. This system was proposed to be used as an in situ system to detect the toxicity of hydrophobic contaminants in soils and for the performance evaluation of PAH degradation in soils. Several biosensors have been developed for the detection of benzene, toluene, ethylbenzene and xylene isomers (89, 273, 363, 668). *E. coli* HB101 cells harboring engineered plasmid pTSN316 (carrying a transcriptional fusion between firefly *luc* genes and the promoter of the *xylS* gene) were immobilized on the tip of a fiber-optic system with a dialysis polycarbonate membrane were able to detect BTEX compounds and related monoaromatics (ethyltoluene and chlorotoluene) in the ppm range (273). The toluene detection range of *E. coli* cells carrying pGLTUR plasmid (fusion of firefly *luc* genes to transcriptional activator *xylR* gene) was between 10 and 20 μM (668). The calculated toluene concentrations were within 3% of those measured by gas chromatography-mass spectroscopy techniques.

To monitor toluene and trichloroethylene cometabolism and kinetics of degradation an on-line monitoring system was developed with *P. putida* B2, which harbors a plamid with *tod-luxCDANE* transcriptional fusion (26, 316). A linear relationship between bioluminescence and toluene concentrations between 0 and 10 mg/liter was observed in assays of *P. putida* B2 growing cells. The cells immobilized in alginate beads were also able to provide on-line monitoring of biotransformation and cometabolism of toluene and trichloroethylene.

Simpson et al. (570) developed an advanced system consisting of biosensor cells interfaced with an intgrated circuit called the bioluminescent bioreporter integrated circuit, which can detect the optical signal, distinguish it from the noise, perform signal processing, communicate the results, and also carry out position sensing. A prototype has been constructed with *P. putida* TVA8 cells with a sensing capacity for toluene vapors at 1 ppm.

A bacterial biosensor for measuring the bioavailable middle-chain-length alkanes was developed (586). *E. coli* DH5α containing the regulatory gene *alkS* and a transcriptional fusion between the *alkB* promoter and *luxAB* genes on two different compatible plasmids was used. The biosensor responded to octane at concentrations as low as 24.5 nM, with a linear response up to 790 nM. The biosensor cells were capable of sensing a range of other compounds that were structurally related, including linear alkanes from pentane to decane and the branched alkane 3-methylheptane.

Even with the rapid advances in nanotechnology, there are still limitations with the bioluminescent bacterial biosensors. Living cells are complex systems, and light output of the bi-

oluminescent biosensors depends not only on the chemical complexity of the sample but also on variations of the physiological state of the cells, including changes in the rate of gene transcription, protein synthesis, membrane permeability, and metabolism. Over the last decade, advances have been made in the use of molecular diagnostics in bioremediation. Qualitative detection methods have been replaced with methods that provide quantitative measurements of specific microbial populations present in the contaminated sites. To assess the microbial treatment of petroleum-contaminated sites, the bioavailabile concentration of pollutants could be measured with bacterial sensors and the overall genetic potential of the degradative pathways determined by DNA tests. It could also be verified whether the pollutant concentrations are sufficiently high to induce the particular degradation. However, the validity of these methods needs to be tested in the field to assess the practicability and usefulness of these techniques in bioremediation. The commercialization of biosensors for environmental applications has shown only modest progress over the last 5 years. The advances in nanotechnology will continue to result in higher sensitivity and more versatile operational characteristics. Nevertheless, whole-cell biosensors hold a great deal of promise for continuous online monitoring of pollutants in environmental applications.

CONCLUSIONS AND FUTURE PROSPECTS

Our review of hydrocarbon metabolism illustrates how molecular tools are contributing to substantially advance our knowledge of the intricate mechanisms of transformation of hydrocarbons. Because of the more challenging methodologies involved in implementing research on anaerobic microbial hydrocarbon degradation, our understanding of this area has lagged behind that of aerobic systems, and great opportunities exist to further elucidate anaerobic hydrocarbon cellular processing mechanisms. These metabolic studies, both aerobic and anaerobic, will in turn provide a greater insight into novel biocatalytic mechanisms.

At least in the context of a perspective that microbes generally thrive in aqueous environments, the hydrophobic nature of hydrocarbons represents a physiological challenge to microbial systems to address hydrocarbon accession. Detailed mechanisms of hydrocarbon uptake and efflux have only recently been reported. Excellent advances in our knowledge of active hydrocarbon efflux, mediated by different efflux pumps, have recently been made. While evidence exists that some of the processes of hydrocarbon uptake are energy dependent, molecular mechanisms for active hydrocarbon uptake have not been established. Further studies in these areas will undoubtedly lead to exciting new findings and add an important dimension to the overall scientific quest to better understand all cellular transport mechanisms.

The biochemical basis of bacterial chemotaxis has been studied for water-soluble systems. However, little is known about taxis as it applies to the mechanisms used by hydrocarbon-degrading bacteria addressing water-insoluble substrates. Nevertheless, preliminary evidence for chemotaxis has been provided, suggesting that exciting opportunities exist to probe the underlying mechanisms involved.

Studies of community dynamics related to petroleum-degrading microbes have the potential, inter alia, to enhance our understanding of the roles played by microbes in the natural genesis of petroleum over geological time and on the long-term effects of petroleum pollution and to determine new remediation and waste treatment approaches. These studies provide insights into the awesome diversity of microbial populations, and accelerated molecular and genomic methodologies and more automated techniques will undoubtedly lead to the characterization of exciting new microbial strains and biocatalytic activities. Apart from adding to our understanding of the complexities of these natural communities, the strains and their metabolic capabilities will surely find new applications in microbial technology.

The bioremediation component of this review focused on treatment of high-volume hydrocarbon wastes. The data show that conventional landfarming of these wastes leaves substantial proportions of the constituent hydrocarbons, including the highly toxic high-molecular-weight PAHs, undegraded. Evidence is also provided that in landfarming practices, as in many conventional bioremediation systems, a large fraction of the volatile hydrocarbons is not biodegraded but is rather transferred to the atmosphere through volatilization. An increasing focus on regulation and control of volatile organic carbon emissions calls for hydrocarbon remediation and waste treatment systems which contain or destroy the volatile organic carbon fraction. These environmental requirements provide scope to microbiologists to establish bioreactor-based environments in which oily soil slurries and sludges may be treated, with volatile organic carbon containment, and where rates and extents of hydrocarbon degradation are maximized. Surfactants can be used to support hydrocarbon accession, and there is evidence that retention of the volatile organic carbons as microbial substrates, rather than their volatilization, facilitates biodegradation of some of the more recalcitrant molecules through cometabolism. This review demonstrates that these more optimized systems greatly accelerate biodegradation processes from the rates observed in landfarms (0.5 to 1% of total petroleum hydrocarbon contents per month) to around 1% per day in large-scale bioreactors while achieving endpoint nonhazardous criteria.

Our knowledge of the potential roles of chemical and biosurfactants in accelerating hydrocarbon accession is still very limited. While bioreactor use facilitates volatile organic carbon containment and process optimization and control, this remediation approach would also enable genetically engineered organisms to be exploited for specific bioremediation applications, given that we are still left with discretion over their fate in the bioreactor-treated material.

Microbial enhanced oil recovery processes mobilize oil in reservoirs through repressurization and viscosity reduction mechanisms. As with in situ bioremediation systems, the environment, over which the microbiologist has little control, influences optimal performance. Clearly, microbial products reducing oil viscosity could be produced above ground under optimal conditions and injected with high chances of efficacy, and research on finding microbial products with universal applications in this area is worth pursuing. A more robust universal microbial system for assisting in the repressurizing of porous reservoirs is desirable and should be aided by ongoing modeling studies directed to manipulating simulated porous

reservoirs in columns. These approaches will facilitate implementation of microbe-based research to determine the most desirable strain types, nutritional, metabolic, and physiological characteristics needed to achieve high success rates in application of the microbial technology in oil recovery. However, the future efficacy of improved microbial enhanced oil recovery technology remains very uncertain.

While hydrocarbon-metabolizing bacteria demonstrate efficient emulsification characteristics (oil-in-water emulsions) during oil biodegradation, microbial deemulsification phenomena are typically observed in oil-dominant water-in-oil systems. Biological deemulsification could provide a low-capital-cost solution for treatment of wellhead emulsions. Current studies indicate substantial performance variation with different emulsion compositions and different oils. Research aimed at understanding the nature of these processes and the critical factors influencing deemulsification is needed.

Microbial processes for desulfurization and denitrogenation of crude oil are limited by the challenges of having to operate in a two-phase aqueous-oil system, and desulfurization efforts have shifted towards reduction of the sulfur content of diesel fuel to achieve the lower prescribed sulfur levels in this product. Recent research has resulted in a 200-fold increase in expression of key desulfurization genes in the best strains. The main challenge now relates to broadening of the specificity of the key biocatalysts, which currently exibit limited activity towards important thiophene and benzothiophene sulfur components.

There is substantial interest in the use of biocatalysis for the synthesis of chiral bioactive compounds and the use of nonconventional aqueous-organic reaction media to accommodate hydrophobic reactants. Recently, the potential for hydrcarbon-transforming oxygenases and other enzymes in enantiospecific biotransformations has been demonstrated. Future research exploiting molecular techniques promises to create a range of novel biocatalysts which could underpin microbial technology for commercial production of high-value enantiomers from petroleum precursors.

On a different front, bacteria with selected petroleum-metabolizing enzymes combined with systems amenable to electronic monitoring, for example, bioluminesence genes, may be used as biosensors to monitor particular petroleum-based environmental pollutants or toxicity in process contol applications. This research topic is at an early stage of investigation but represents an important interdisciplinary pursuit encompassing biological and electronic expertise.

Clearly, petroleum microbiology research is advancing on many fronts, spurred on most recently by new knowledge of cellular structure and function gained through molecular and protein engineering techniques, combined with more conventional microbial methods. Improved systems for biodegradation of petroleum components are being commercialized with positive economic and environmental advantages. Groundbreaking work is being done to engineer new biocatalysts for applications in desulfurization and organic synthesis.

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